UNIVERSITY OF COPENHAGEN

Salmonella Dublin in cattle

epidemiology, design and evaluation of surveillance and eradication programmes

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FACULTY OF HEALTH AND MEDICAL SCIENCES
UNIVERSITY OF COPENHAGEN

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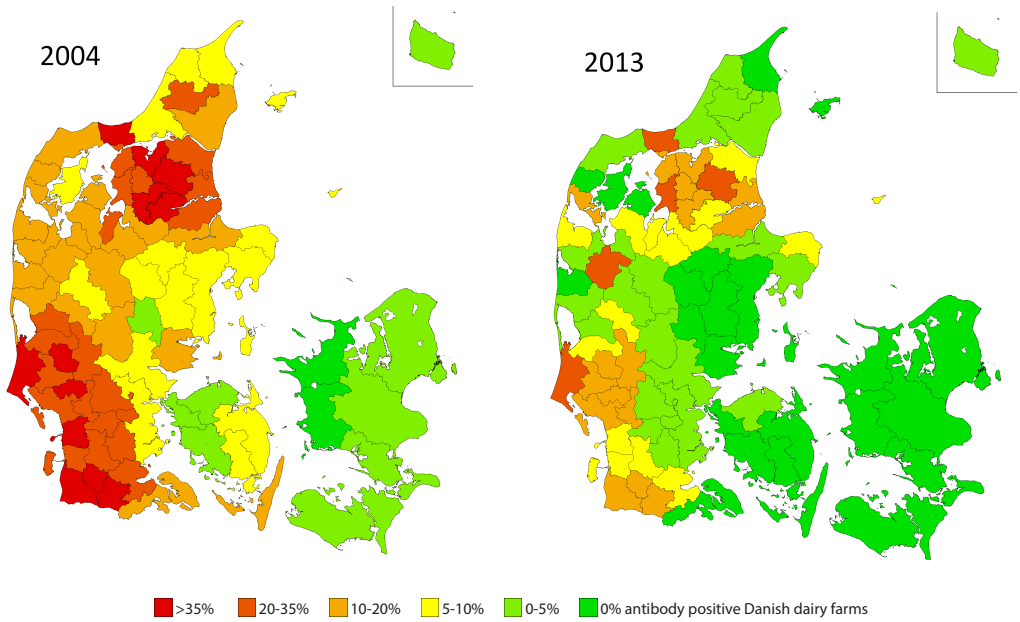
Dr. med. vet. thesis 2013

LIZA ROSENBAUM NIELSEN



Salmonella Dublin in cattle

Epidemiology, design and evaluation of surveillance and eradication programmes

Dr. med. vet. thesis

Liza Rosenbaum Nielsen

"Let's hope it's not salmonella"

Department of Large Animal Sciences · Faculty of Health and Medical Sciences
University of Copenhagen · Denmark · 2013

Denne afhandling er af Det Sundhedsvidenskabelige Fakultet
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Preface

Salmonella bacteria are among the most frequently diagnosed causes of foodborne disease in humans worldwide. They are also commonly present in production animal populations unless effective surveillance and control efforts are on-going to reduce and prevent spread of the infections. Thus, *Salmonellae* are high on lists of zoonotic infections requiring centrally organised surveillance and control programmes. Denmark currently has such programmes in place for broilers and layers, pigs and pork, and cattle and beef.

In Denmark, and several other countries with similar production systems for dairy and beef, the two most commonly isolated *Salmonella* (*S*.) serovars in cattle are *S*. Dublin and *S*. Typhimurium. *S*. Dublin differs from other *Salmonella* serovars by being host-adapted to cattle. It leads to enteric and systemic disease in affected bovines and has been associated with increased morbidity, mortality and production losses in infected herds. Up through the 1980'ies and 1990'ies research suggested that *S*. Dublin has a tendency to become persistent in some infected animals and herds. Therefore, there was a growing demand for studies that lead to a better understanding of *S*. Dublin pathogenesis and epidemiology both at animal and herd level to understand how to combat this infection effectively.

The Danish cattle industry together with the Danish Veterinary and Food Administration initiated a national surveillance programme for *S.* Dublin in all cattle herds in 2002. As a consequence, there was a marked decrease in prevalence from approximately 25% to 16% infected dairy herds between 2002 and 2005. Studies presented and discussed in this thesis cover research regarding diagnostic test-strategies and scientific decision support required to design and evaluate the performance and expected outcomes of the surveillance and eradication programmes. This led to adjustments of the surveillance programme in 2006 and 2010. To achieve further reductions in prevalence after 2006, a more intensive control programme that was initiated in 2007 with the aim to eradicate *S*. Dublin from the Danish cattle population.

Hence, this thesis provides background and summarises essential epidemiological studies that have been used to support the design and development of the Danish surveillance and eradication programmes for *S.* Dublin in cattle. The thesis is built up around questions that I was frequently asked between 2003 and 2012 concerning control of *S*. Dublin. I hope that it will be useful for other countries wishing to control this infection effectively and efficiently.

Liza Rosenbaum Nielsen
DVM, PhD, DipECVPH

Frederiksberg, June 23rd 2013

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The research presented in this thesis was mainly performed at the Department of Large Animal Sciences, Faculty of Health and Medical Sciences at University of Copenhagen, Denmark (prior to 2007 The Royal Veterinary & Agricultural University; between 2007-2011 'Faculty of Life Sciences' at University of Copenhagen). I wish to thank all current and previous students and colleagues who have inspired, helped, constructively criticised and encouraged me throughout the years since I was first employed there in year 2000. Special thanks to Professor Hans Houe and Professor Søren Saxmose Nielsen for engaged critical reading of this thesis. No other names mentioned, no other names forgotten.

The same gratitude goes to the current and previous colleagues at the Danish Cattle Federation (previously at the Danish Dairy Board), AgroTech A/S and the Danish Agriculture & Food Council without whom this work would not have been performed. The collaboration with the dairy and beef industries has been uniquely positive in many ways.

I take the opportunity to thank the Danish Cattle Federation, the Danish Dairy Board, the Milk Levy Fund, the Cattle Levy Fund, the Ministry of Food, Agriculture and Fisheries, the Danish Food Industry Agency (DFFE: Journal nr. 3412-06-01634), the National Veterinary Institute and the National Food Institute at Danish Technical University, and the Danish Veterinary & Food Administration for funding and supporting the research I was involved in from 2000-2012 in the field of *Salmonella* Dublin epidemiology. The funding and collaboration allowed me to gather the information and synthesise the knowledge presented in this thesis. I also wish to thank 'San Cataldo Fonden' for funding a stay at the retreat San Cataldo in Italy in August 2011 dedicated to the writing of parts of this thesis, and I thank 'Fonden af 13. Juli 1873 til veterinærvidenskabens fremme' for funding a stay at University of Prince Edward Island in September 2011 dedicated to epidemiological analyses of Danish surveillance data.

I wish to express sincere thanks to colleagues in the two International EpiLab projects that I had the pleasure of being project leader of between 2004 and 2007, not least the two international guest scientists Dr. Lorin Warnick from College of Veterinary Medicine, Cornell University, New York State, USA and Dr. David Jordan from Department of Primary Industries, New South Wales, Australia. Sincere thanks also go to Dr. Ian Dohoo from Atlantic Veterinary College, Prince Edward Island, Canada, for generous help with epidemiological analyses and very helpful discussions about several of the manuscripts in this thesis. I have learned a lot these great scientists.

Many thanks also go to colleagues at Research Centre Foulum at Aarhus University, Animal Health Service in Deventer and Utrecht University in The Netherlands for collaboration on different projects, and thanks to several European and North American colleagues for fruitful discussions about *Salmonella* surveillance and control over the years.

Special thanks go to Karsten Aagaard, former veterinary chief at the Danish Cattle Federation. Without his visionary line of thinking, his ability to initiate research and use the results, and his determination to set goals and reach them, Denmark would not have been this far with the *S*. Dublin programmes, and I would not have had the opportunity to write this thesis.

Last, but definitely not least, lots of thanks to my dear family and good friends, who encouraged me and put up with me, to Hans and Tove for letting me occupy the summer house a lot during the intensive writing periods, and to Peter for being the music of my life.

Liza Rosenbaum Nielsen, Frederiksberg 2013

PART 1: DOCTORAL THESIS

English summary

Background

Salmonella enterica subsp. *enterica* serovar Dublin (*S.* Dublin) is a bacterium that is host-adapted to cattle. *S.* Dublin causes both acute and chronic clinical disease, as well as subclinical and transient or persistent latent infections in exposed cattle. It has received a lot of attention in the Danish cattle industry and from veterinary authorities since the beginning of the 1990's due to its adverse effects on animal health, welfare and economic losses in infected herds. Outbreaks in other species are also seen from time to time, e.g. in mink, pigs or horses, but usually such infections can be traced back to a cattle source. In addition, it is a severe zoonotic infection. In 2011, 42 (3.6%) of all of the reported human salmonellosis cases in Denmark were caused by *S*. Dublin. An increasing proportion of the human cases in Denmark has been attributed to imported beef or has been acquired abroad. In 2011, six human cases were attributed to domestically produced beef, but the numbers are uncertain. Human cases are characterised by septicaemia and a high case fatality risk.

Studies in the mid 1990s estimated that as much as one fifth of the dairy herds were infected. Research performed from 1998 to 2003 led to a better understanding of the accuracy of the available diagnostic tests at animal and herd level, and in October 2002 a national surveillance programme in all cattle herds was initiated. The aim of the programme was to estimate and monitor the prevalence of *S*. Dublin in the cattle population over time, and to provide farmers with tools to prevent an introduction of *S*. Dublin when purchasing animals. This led to immediate dramatic changes in trade patterns, and the prevalence of test positive dairy herds was reduced from approximately 26% to 16% between 2002 and 2005. Based on the results from studies, evaluating the surveillance programme and some modelling of the effect of the different control scenarios in the dairy sector, important adjustments of the surveillance followed in 2006, and a more intensive control programme was initiated in 2007 with the aim to eradicate *S*. Dublin from the Danish cattle population.

Aim of thesis

The aim of this thesis was to produce new evidence-based knowledge about *S*. Dublin epidemiology including information about its occurrence and infection dynamics. This is of high importance for the design and evaluation of rational and effective surveillance and eradication programmes for *S*. Dublin in cattle populations. The material mainly covers studies performed between 2000 and 2012, and also includes a review of pathogenesis and diagnosis. The studies were used to support the development of the Danish *S*. Dublin programmes and to provide appropriate decision support for farmers who wish to eradicate the infection from their herds. The work is described and discussed in eight chapters supported by 16 accompanying papers that have been published or submitted for publication in international peer-reviewed journals.

Pathogenesis

A review paper (Paper I, Chapter 2) has been included that concerns the current understanding of *S.* Dublin pathogenesis and diagnosis. This is needed to perform and understand the epidemiological studies described in chapters 3 to 8. In short, knowledge about *S*. Dublin pathogenesis is based mostly on experimental studies and an understanding of the disease mechanisms at a cellular level.

The mechanisms for host-adaptation are not well understood. However, the fact that S. Dublin is host-adapted facilitates the control of the infection within the cattle sector. Susceptibility to *S*. Dublin decreases with age, both with respect to intestinal colonisation, invasion and the clinical consequences of exposure. Sufficiently large doses will affect all ages of the cattle, and co-infections such as bovine virus, diarrhoea virus, liverflukes (*Fasciola hepatica*) and other types of stress can reduce the host's resistance to the bacteria, regardless of age. The most common route of infection is faecal-oral transmission. The bacteria can colonise and invade the host's cells within 6 hours and be shed in faeces in less than 24 hours, which makes *S*. Dublin a fast-spreading infection under the right conditions. Invasiveness varies between the different *S.* Dublin strains. Cell-mediated immunity is more important for the protection of cattle against *S.* Dublin than antibody responses. Antibodies are, however, useful for diagnostics as described below, but do not provide a sufficient immunity for the animal against these highly invasive and intracellular bacteria.

Diagnosis

Currently no sensitive bacteria-detecting tests are available on the market. This would be useful for a rapid and accurate outbreak diagnosis, especially towards the end of an eradication campaign. This would also be useful for research purposes, in particular if it was possible to analyse many samples simultaneously at a low cost. Antibody responses to *S*. Dublin in individual cattle upon an uptake of the bacteria can be measured, e.g. as immuno-globulin-G (IgG) responses using enzyme-linked immunosorbent assay (ELISA). The IgG level begins to increase at around 2 weeks after an uptake of the bacteria in calves that are older than 3 months. Younger calves have a poorer antibody response, but may have maternally derived serum antibodies that wane during the first month and provide little protection. Older cattle can have a faster primary IgG response to infection. The maximum IgG titre is reached at between 6 and 11 weeks after the uptake of the bacteria, and gradually decreases to reach baseline levels between 2 and 3 months after a peak IgG titre, unless the animal is exposed to the bacteria again. Under natural farm conditions repeated exposure of cattle in infected herds is common. This leads to less clear serological patterns in individual animals than under controlled experimental settings. Therefore, the use of serology for a diagnosis of the infection status of individual animals is tricky. Instead, easy, cost-effective group diagnostics can be used for risk reduction, when ELISA is a part of the control strategies in cattle herds. Antibody measurements on bulk-tank milk (BTM) samples are cheap and effective tools in the surveillance and control programmes as demonstrated in this thesis.

Prevalence in dairy and veal calf herds

Studies on a within-herd and herd level prevalence, and incidence estimations of *S*. Dublin in dairy herds and specialised veal calf herds are presented. The within-herd prevalence varied between herds and age groups. Whereas the overall animal level prevalence was 1.3% in veal calves that were delivered to slaughter, the within-herd estimated true prevalence of culture positive cattle from the infected veal calf herds varied between 4.8% and 24.5%. This was comparable to the within-herd faecal culture prevalence that was found in calves in 14 *S*. Dublin infected dairy herds that were tested intensively over a 1-year period. For comparison, the within-herd true prevalence estimates from a Bayesian analysis of both serological and faecal culture data from the infected veal calf herds varied from 21% to 49%, i.e. 2-4 times higher than when the estimates were based on the faecal culture only. This emphasises the importance of taking into account the diagnostic methods and accuracy when estimating prevalence. In endemically infected dairy herds, the seroprevalence was

on average 15%-35% in most groups of calves, heifers and adult cows. This is higher than what was reported previously in studies from other countries.

Large spatiotemporal differences were evident for a herd level true prevalence ranging from more than 35% in high prevalence regions at the beginning of the surveillance programme in 2002, to close to zero in low prevalence regions in 2012. Prevalence was highly correlated with cattle density. Herd level prevalence estimates differed markedly between two studies of specialised veal calf herds. The difference could be attributed to the methods used for the prevalence estimations. In the study based on a Bayesian estimation using both faecal culture results and serological results from the calves in the study herds, the herd level prevalence was estimated at between 34% and 57%, i.e. higher than the 18% estimated in a study relying only on bacteriological cultures.

All in all, the studies in this thesis contribute with a much more detailed knowledge of the occurrences of *S*. Dublin in veal calves and dairy cattle herds than has previously been available. This knowledge is important in the planning of optimal test strategies, surveillance and control programmes in cattle herds.

Risk factors for introduction and persistence

Risk factor studies of *S*. Dublin introduction and persistence in dairy herds, and a risk factor study of the occurrence of *S*. Dublin in veal calf herds are presented in this thesis. The most consistent risk factors for both the introduction and the persistence of *S*. Dublin were purchase of cattle from test positive cattle herds, local prevalence level or the number of test positive herds within a 2 or 5 km radius and herd size.

While organic and conventional production was associated with a similar risk of an introduction of *S*. Dublin in dairy herds, a higher tendency to persist in infected, organic herd was revealed in two risk factor studies. Increasing somatic cell counts in bulk-tank milk increased the risk of an introduction and reduced the probability of a recovery meaning that it favoured the persistence of infection. Interestingly, a potential synergetic effect between the participation in the voluntary paratuberculosis control programme and an improved control of *S*. Dublin was found. Herds enrolled in the paratuberculosis programme had markedly shorter durations of *S*. Dublin infections than those not enrolled when local prevalence was high. This association was not relevant in low prevalence regions, which suggests that it is related to improved external biosecurity in the enrolled herds. July to October was high season for the introduction of infection, whereas recovery was not seasonal. The results emphasised the importance of movement restrictions, as well as external and internal biosecurity measures to prevent and control *S*. Dublin infections at both herd and national level, but also suggested that more knowledge is needed regarding the more diffuse transmission pathways in regions with many infected herds and high cattle density. The studies in this thesis distinguish themselves from the few previous risk factor studies in the literature by being based on markedly larger and longitudinal data sets, which facilitated more robust statistical and epidemiological analyses of the risk factors in question.

Infection dynamics

Results from an age-structured, stochastic and mechanistic simulation model, 'Dublin-Simherd', of the within-herd infection dynamics and the effects of *S*. Dublin in dairy herds are presented in two papers in this thesis. The model uses an object-based approach to model infection dynamics within virtual Danish dairy herds. Herd size, hygiene and management levels were highly influential on

epidemic size, duration of infection and the probability of extinction within a simulated 10-year period. Economic effects of *S*. Dublin introduction and a subsequent spread of infection were substantial, e.g. an average of 49,000 euro (245 euro per cow stall) were estimated to be lost in gross margins during the first year in a herd with 200 cow stalls and poor management. An average annual loss of 30,200 euro (151 euro per cow stall) was estimated over the 10-year period following introduction of infection. The estimated economic losses were mainly due to milk yield losses in infected and recently infected (resistant) cows, and were highly correlated with the epidemic sizes and durations of infections, which again were highly correlated with the level of hygiene and management in the herd. Thus, the economic losses of *S*. Dublin infection estimated here are markedly higher than previously described, among other things because the estimation method includes effects that go unnoticed by many farmers, consultants and researchers.

A similar object-based approach to the simulation of national control strategies in dairy herds was used in another study also presented in this thesis. The model was used for decision support in the design of the Danish control and eradication programme from 2007 to 2014. More frequent testing of bulk-tank milk for antibodies to *S*. Dublin was shown to be less effective than an improved herd biosecurity. It was also found that restricting cattle movement between regions provided a strong benefit to those regions initially with a low prevalence of infection, but made it more difficult for high prevalence regions to reduce their prevalence of infection. Enhanced control through improved biosecurity within infected herds was of intermediate benefit, and a combination of strategies was highly effective in obtaining a low prevalence over a period of 10 years, although the cost and feasibility of this option need further exploration. An added benefit of the object-based modelling method used in this thesis, which differs from the more traditional mathematical modelling approaches used in studies in the literature, was that the intuitive model structure facilitated communication of the results to the stakeholders, an aspect which is sometimes underestimated as a prerequisite of a successful outcome.

Diagnostic test-strategies and surveillance

Several studies in this thesis cover different aspects of diagnostic test strategies, design and evaluation of surveillance programmes for *S*. Dublin in cattle herds. Testing to determine the infection status of individual cattle is unfortunately difficult. Employing an assessment of progress concept on groups of animals rather that interpretation of repeated individual antibody measurements as previously recommended is important in the interpretation and use of animal level test results. Faecal culture generally has very poor diagnostic sensitivity in naturally infected cattle, and should really only be considered for a confirmation of infection in clinically ill, non-treated animals or if deemed necessary for confirmation of suspected faecal shedders in a group of animals that keep having very high levels of antibodies in a high proportion of the animals. The previously recommended method of carrier detection, based on two or three high antibody measurements with 90 to 120 days in between, was evaluated in this thesis and was not found sufficiently predictive of high bacterial shedding probabilities in animals in endemically infected dairy herds. Less than 30% of the cattle with such temporal antibody profiles posed a risk of spreading the infection, and the highest risk (~5%) was found in cattle < 2 year old with a recently high or repeatedly high antibody measurement. This complicates test-and-cull strategies as a tool in control and eradication programmes for *S*. Dublin. In adult cattle > 3 years old, the probability of a positive faecal culture was found to be low (<2%) regardless of the measured antibody levels or temporal antibody profiles.

For herd diagnoses test-strategies based on antibody measurements were generally more sensitive than test-strategies based on bacteriological culture. The most sensitive test strategy reported in a previous study was a combination of bulk-tank milk antibody measurements and the serology of calves between 4-6 months of age. The test strategy used in the Danish surveillance programme which bases herd classification on the average of four consecutive bulk-tank milk antibody measurements automatically collected at year-quarterly intervals, had comparable sensitivity and specificity at much lower costs. Improvements to the predictive values of the sampling scheme might be obtained through early warning systems in low-risk herds and follow-up diagnostics in test positive herds.

A compromise between sensitivity, specificity and cost is inevitable when selecting a sampling strategy for the surveillance of *S*. Dublin based on the blood sampling of non-dairy cattle. The sample sizes of 8 to 10 animals used in the Danish surveillance and control programmes for different purposes is a sensitive herd testing strategy. However, if only one animal has a high ELISA response, it might be either because of a very low prevalence, which is particularly unlikely in outbreak situations, or a false positive reaction that warrants follow-up investigations in the herd. A reasonable alternative test strategy might be to set the criterion for a test positive herd diagnosis to two out of 8-10 seropositive samples from the herd. This would lead to few false positive herd classifications and would still allow for detection of the infectious herds with reasonable sensitivity.

Control and eradication of S. Dublin

All of the 16 accompanying papers provided with this thesis have led to new knowledge and scientific evidence to support decisions about approaches and methods to go by nationally, or have provided recommendations to farmers who want to control the infection in their herds. One paper is specifically concerned with control at a national level. The recommendation was used to adjust the control elements implicit in the national surveillance programme in 2006 and a more intensified control and eradication campaign followed in late 2007 focusing on the external and internal biosecurity of infected herds. Suggestions to combine these efforts with regional movement restrictions and stricter movement restrictions out of infected or test positive herds have since been discussed and are currently being implemented.

Two other papers were aimed at investigating and demonstrating a structured approach to the control of *S*. Dublin in infected cattle herds and at using test-and-culling practices in 10 dairy herds that tried to eradicate the infection from their cattle herd over a 3-year period. There was evidence that the chosen control elements were herd specific, and worked for the nine participating herds that did not purchase cattle from test positive herds. Culling of repeatedly antibody positive cows was only practical for farmers, when the prevalence was reduced markedly in the herds, and when there was evidence that transmission of infection had ceased among the calves and young stock.

Conclusions and perspectives

The work presented in this thesis provides scientific evidence that the required technical, epidemiological and economic preconditions for the eradication of *S*. Dublin exist in Denmark, and how it can be achieved in an effective way. Having a large livestock industry and high standards for food safety, animal health and welfare, Denmark should be able to provide the administrative, operational and financial resources required to eradicate *S*. Dublin. The steps taken so far strongly indicate that this is the case. It is difficult to see how the Danish approach to the eradication of *S*.

Dublin can be hampered by socio-ecological factors other than potential counteractive perceptions, attitudes and behaviours among some of the reluctant farmers and local veterinarians and cattle advisors. In this thesis it was described how such perceptions and behaviours can be influenced not only by legislation, but also through participatory field projects for owners or managers of infected herds who are in need of support to carry out the required control efforts. Many of the approaches used in Denmark and described here could be readily and advantageously implemented in other countries with milk and beef production and prevalent *S*. Dublin infections.

Future studies are suggested to focus on clarifying the impact and causes of diffuse contamination and infection pathways that may impede the success of the programme, such as the significance of transportation and the spread of contaminated manure, large flocks of migrating birds, access to and contamination of surface water. Furthermore, it is recommended that the occurrence and potential sources of other *Salmonella* serotypes in the Danish cattle herds are investigated. A potential increase in new *Salmonella* serotypes may lead to new disease patterns in infected herds and cross-reactions in the antibody tests in the surveillance programme. Finally, due to difficulties in controlling *S*. Dublin in some types of herds it might be worth evaluating alternative control scenarios in specific herds with exceptional conditions leading to persistence of infection, e.g. very large herds, organic herds, multi-site herds etc.

Resumé på dansk

Baggrund

Salmonella enterica subsp. *enterica* serovar Dublin (*S*. Dublin) er en bakterie, der er værtstilpasset kvæg. *S*. Dublin forårsager både akut og kronisk klinisk sygdom, såvel som subkliniske og forbigående eller vedvarende latente infektioner i kvæg, der udsættes for smitten. Bakterien har fået stor opmærksomhed i den danske kvægindustri og de veterinære myndigheder siden begyndelsen af 1990'erne på grund af dens negative indvirkning på dyrs sundhed, velfærd og de økonomiske tab i inficerede besætninger. Udbrud i andre arter ses også fra tid til anden, fx i mink, svin eller heste, men normalt kan infektioner i andre arter end kvæg spores tilbage til en kvægkilde. Desuden er *S*. Dublin en alvorlig zoonose. I 2011 blev 42 (3,6 %) af alle de rapporterede humane salmonellatilfælde i Danmark forårsaget af *S*. Dublin. En stigende andel af de humane tilfælde i Danmark er blevet tilskrevet importeret oksekød eller er erhvervet i udlandet. I 2011 blev seks humane tilfælde tilskrevet dansk produceret oksekød, men tallene er behæftet med stor usikkerhed. Humane tilfælde er karakteriseret ved sepsis og en høj dødelighed hos mennesker, der registreres med infektionen.

Studier fra midten af 1990'erne anslog at omkring en femtedel af malkekvægsbesætningerne var smittede med *S*. Dublin. Forskning udført 1998-2003 førte til en bedre forståelse af validiteten af de tilgængelige diagnostiske tests på dyre-og besætningsniveau, og i oktober 2002 blev et nationalt overvågningsprogram i alle kvægbesætninger påbegyndt. Formålet med programmet var at vurdere og overvåge forekomsten af *S*. Dublin i kvægbestanden over tid, samt at give landmænd værktøjer til at forhindre en introduktion af *S*. Dublin til deres besætning, når de køber dyr. Dette førte til øjeblikkelige dramatiske ændringer i handelsmønstre, og forekomsten af testpositive malkekvægsbesætninger blev reduceret fra ca. 26 % til 16 % mellem 2002 og 2005. Baseret på resultaterne fra undersøgelser, der evaluerede overvågningsprogrammet og modellerede effekten af forskellige kontrolscenarier i malkekvægssektoren, blev der gennemført vigtige justeringer af overvågningsprogrammet i 2006, og en mere intensiv bekæmpelseskampagne blev indledt i 2007 med det formål at udrydde *S*. Dublin fra den danske kvægbestand.

Formål med afhandlingen

Formålet med denne afhandling er at producere ny evidensbaseret viden om *S*. Dublin epidemiologi herunder oplysninger om *S*. Dublins forekomst og infektionsdynamik. Det er af stor betydning for udformning og evaluering af et rationelt og effektivt overvågnings- og udryddelsesprogram for *S*. Dublin i kvægpopulationen. Materialet dækker hovedsageligt undersøgelser udført mellem 2000 og 2012, og omfatter også en gennemgang af patogenese og diagnostik. Undersøgelserne blev brugt til at understøtte udviklingen af de danske *S*. Dublin programmer og til at bidrage med beslutningsstøtte for landmænd, der ønsker at udrydde infektionen fra deres besætninger. Arbejdet er beskrevet og diskuteret i otte kapitler understøttet af 16 medfølgende artikler, der er publiceret i internationale peer-reviewede tidsskrifter.

Patogenese

En oversigtsartikel (Paper I, kapitel 2) som vedrører den nuværende viden af *S*. Dublins patogenese og diagnostik er medtaget. Den viden var nødvendig for at udføre og forstå de epidemiologiske studier beskrevet i kapitel 3 til 8. Kort sagt er viden om *S*. Dublin patogenese hovedsageligt baseret

på eksperimentelle studier og en forståelse af sygdomsmekanismer på celleniveau. Mekanismerne for værtstilpasning er ikke klarlagt, men den omstændighed, at *S*. Dublin er værtstilpasset, gør det nemmere at bekæmpe den i kvægsektoren. Modtagelighed for *S*. Dublin falder med alderen, både med hensyn til kolonisering af tarmen, invadering af værten og de kliniske konsekvenser af eksponering. Tilstrækkeligt store doser vil dog påvirke alle aldre af kvæg og andre infektioner såsom bovin virus, diarré virus, leverikter (*Fasciola hepatica*) og andre typer af stress kan reducere værtens modstandsdygtighed over for bakterierne, uanset alder. Den mest almindelige smittevej er fækal-oral transmission. Bakterierne kan kolonisere og invadere værtens celler inden for 6 timer og udskilles i fæces efter mindre end 24 timer, hvilket gør *S*. Dublin en hurtigspredende infektion under de rette betingelser. Evnen til at invadere værten varierer mellem forskellige *S*. Dublin stammer. Cellemedieret immunitet er mere vigtigt for beskyttelsen af kvæg mod *S*. Dublin end antistofreaktioner. Antistoffer er imidlertid nyttige til diagnostik som beskrevet nedenfor, men giver ikke en tilstrækkelig immunitet for dyret mod disse meget invasive, intracellulære bakterier.

Diagnose

Der findes endnu ingen følsomme testmetoder til bakteriepåvisning på markedet. Dette ville ellers være nyttigt for at få en hurtig og præcis diagnose, især hen mod slutningen af et udryddelses-program. Det ville også være nyttigt til forskningsformål, især hvis det var muligt at analysere mange prøver samtidigt til en lav pris. Antistofrespons på *S*. Dublin i enkeltdyr efter optag af bakterier kan måles, f.eks. som immunoglobulin G (IgG)-respons ved hjælp af 'enzymelinked immunosorbent assay' (ELISA). IgG-niveauet begynder at stige omkring 2 uger efter en optag af de bakterier, hos kvæg der er ældre end 3 måneder. Yngre kalve har et dårligere antistofrespons, men kan i serum have maternelle antistoffer, der aftager i løbet af den første måned og giver lidt beskyttelse. Ældre kvæg kan have et hurtigere primært IgG-respons på infektionen. Den maksimale IgG-titer nås mellem 6 og 11 uger efter optagelsen af bakterierne, og aftager gradvist til tæt på baseline niveauet mellem 2 og 3 måneder efter at IgG-titeren toppede, medmindre dyret bliver udsat for bakterierne igen. Under naturlige staldforhold vil der typisk ske gentagen eksponering af kvæget i smittede besætninger. Dette fører til mindre klare serologiske mønstre i de enkelte dyr end under kontrollerede eksperimentelle forsøg. Derfor er brugen af serologi til diagnosticering af infektionsstatus af det enkelte dyr vanskelig. I stedet kan omkostningseffektiv gruppediagnostik anvendes til at mindske smittespredningsrisikoen, når serologi er en del af kontrolstrategien i kvægbesætninger. Antistofmålinger på tankmælksprøver er, som vist i denne afhandling, billige og effektive værktøjer i overvågnings- og kontrolprogrammer for *S*. Dublin

Prævalens i malkekvægs- og slagtekalvebesætninger

Undersøgelser af prævalensen indenfor og mellem malkekvægsbesætninger og specialiserede slagtekalvebesætninger, samt incidensen af *S*. Dublin i malkekvægsbesætnigner præsenteres her i afhandlingen. Dyreniveau-prævalensen varierede mellem besætninger og aldersgrupper. Mens den overordnede prævalens af slagtekalve, der udskilte bakterier i gødningen på slagteriet var 1,3 % varierede forekomsten i dyr fra smittede slagtekalvebesætninger mellem 4,8 % og 24,5 %. Det er sammenligneligt med forekomsten bakterieudskillere blandt kalve i 14 *S*. Dublin-smittede malkekvægsbesætninger, der blev testet intensivt i løbet af en 1-årig periode. Til sammenligning blev den sande prævalens fra en Bayesiansk analyse af både serologiske og bakteriologiske laboratorieanalyser fra smittede slagtekalvebesætninger estimeret til at variere fra 21 % til 49 %, dvs. 2-4 gange højere, end når estimatet var baseret bakteriologisk dyrkning. Dette understreger vigtigheden af at tage hensyn til de diagnostiske metoders nøjagtighed, når man vurderer

forekomsten af *S*. Dublin i en population. I endemisk smittede besætninger var seroprævalensen gennemsnitligt 15 % -35 % i de fleste grupper af kalve, kvier og voksne køer. Dette er højere, end der blev rapporteret i tidligere undersøgelser fra andre lande.

Der blev fundet store spatiotemporale forskelle i besætningsprævalensen af *S*. Dublin, rangerende fra mere end 35 % i regioner med høj forekomst i starten af overvågningsprogrammet i 2002 til tæt på nul i regioner med lav forekomst i 2012. Udbredelsen var stærkt korreleret med kvægtætheden. Prævalensestimater på besætningsniveau afveg markant mellem to undersøgelser af specialiserede slagtekalvebesætninger. Forskellen kunne tilskrives de anvendte metoder til estimering af forekomsten. I undersøgelsen baseret på Bayesiansk analyse ved hjælp af både bakteriologisk undersøgelse af gødningsprøver og serologiske analyser af serum fra kalve i blev prævalensen estimeret til mellem 34 % og 57 %, dvs. højere end de 18% estimeret i en den undersøgelse, der kun byggede på bakteriologiske undersøgelser af gødningsprøver.

Alt i alt bidrager studierne i denne afhandling med en langt mere detaljeret viden om forekomsten af *S*. Dublin i slagtekalve og malkekvægsbesætninger end der tidligere har været tilgængeligt. Denne viden er vigtig i planlægningen af optimale teststrategier, samt overvågnings- og kontrolprogrammer kvægbesætninger.

Risikofaktorer for introduktion og persistens

Risikofaktorstudier af *S*. Dublin-introduktion og persistens i malkekvægsbesætninger og et risikofaktorstudium af forekomsten af *S*. Dublin i slagtekalvebesætninger præsenteres i denne afhandling. De mest konsistente risikofaktorer, som viste sig at øge både introduktionsrisikoen og persistensen af *S*. Dublin var indkøb af kvæg fra testpositive kvægbesætninger, høj forekomst af *S*. Dublin i lokalområdet (eller højt antal testpositive besætninger inden for en 2 hhv. 5 km radius) og stigende besætningsstørrelse. Økologisk og konventionel produktionsform var forbundet med lige stor risiko for introduktion af *S*. Dublin i malkekvægsbesætninger. Derimod blev der fundet en højere tendens til persistens af infektionen i økologiske besætninger i to risikofaktorerstudier. Stigende celletal i tankmælken gav øget risiko for både introduktion og persistens, hvilket formentlig skyldes underliggende hygiejne- og managementfaktorer. Interessant nok blev en potentiel synergieffekt mellem deltagelse i det frivillige bekæmpelsesprogram for paratuberkulose og bekæmpelse af *S*. Dublin påvist. Besætninger, der deltog i paratuberkuloseprogrammet havde markant kortere varighed af *S*. Dublin-infektioner end dem, der ikke deltog, når den lokale prævalens af *S*. Dublin var høj. Denne effekt var tilsyneladende ikke relevant i lavprævalente regioner, hvilket tyder på, at det er relateret til forbedret smittebeskyttelse i de besætninger, der deltog i paratuberkuloseprogrammet. Juli til oktober var højsæsonen for nysmitte med *S*. Dublin, mens det at skifte fra smittet til usmittet niveau i overvågningsprogrammet ikke var sæsonbestemt. Resultaterne understreger betydningen af målrettede flytterestriktioner, samt anden ekstern og intern smittebeskyttelse for at forebygge og bekæmpe *S*. Dublin-infektion både i smittede besætninger og på nationalt plan. De viser også, at mere viden er nødvendig med hensyn til de mere diffuse smitteveje i regioner med mange smittede besætninger og høj kvæg tæthed. Undersøgelserne i denne afhandling adskiller sig fra tidligere risikofaktorstudier ved at være baseret på særdeles store og langvarige dataindsamlinger, hvilket gjorde det muligt at udføre mere robuste statistiske og epidemiologiske analyser af de pågældende risikofaktorer.

Infektionsdynamik

Resultater fra en aldersstruktureret, stokastisk og mekanistisk simuleringsmodel, 'Dublin-SimHerd', om infektionsdynamikken i malkekvægsbesætninger og virkningerne af *S*. Dublin i malkekvægsbesætninger præsenteres i denne afhandling. Modellen anvender en objekt-baseret tilgang til at modellere infektionsdynamik indenfor virtuelle danske malkekvægsbesætninger. Besætningsstørrelse, hygiejne- og managementniveauer havde stor indflydelse på størrelsen og varigheden af udbrud og på sandsynligheden for at infektionen blev udryddet inden for en simuleret 10-årig periode. Økonomiske virkninger af *S*. Dublin-introduktion og efterfølgende smittespredning var betydelig, fx blev et gennemsnit på 49.000 euro (245 euro per sengebås i kostalden) estimeret til at være tabt i dækningsbidraget i løbet af det første år i en besætning med 200 køer og ringe management. Et gennemsnitlig årligt tab på 30.200 euro (151 euro per sengebås i kostalden) blev estimeret over den 10-årige periode efter nysmitte med S. Dublin i samme type besætning. De forventede økonomiske tab var primært på grund af tab i mælkeydelsen i nysmittede køer og køer, der for nyligt var kommet sig ovenpå infektionen. Størrelsen på de økonomiske tab var stærkt korrelerede med størrelse og varighed af udbrud og efterfølgende smitteperiode i besætningen, hvilket igen var stærkt korreleret med niveauet af hygiejne og management i besætningen. Således er de økonomiske tab af *S*. Dublin-infektion, der blev estimeret her, markant højere end tidligere beskrevet. Dette skyldes blandt andet at beregningsmetoden omfatter effekter, der ikke umiddelbart bliver bemærket af mange landmænd, konsulenter og forskere.

En lignende objekt-orienteret tilgang til simulering af de nationale kontrolstrategier i malkekvægs-besætninger blev brugt i et andet studium, der også præsenteres i denne afhandling. Modellen blev brugt til beslutningsstøtte i udformningen af det danske bekæmpelses- og udryddelses-program i 2007-2014. Hyppigere målinger af tanksmælkantistoffer mod *S*. Dublin viste sig at være mindre effektive end forbedret smittebeskyttelse i den nationale strategi. Det blev også konstateret, at en begrænsning af flytninger af kvæg mellem regioner var en stor fordel for de regioner, der i første omgang havde en lav forekomst af infektionen, men gjorde det vanskeligere at bekæmpe infektionen i regioner med høj forekomst. Forbedret smittebeskyttelse i smittede besætninger rangerede middelhøjt som national strategi, mens en kombination af tiltag var meget effektiv til at opnå en lav prævalens i løbet af en 10-årig periode. Der er dog behov for at undersøge, hvilke omkostninger og realiserbarhed denne mulighed har.

En fordel ved den objekt-baseret modelleringsmetode, der blev brugt i denne afhandling, og som adskiller sig fra de mere traditionelle matematiske modelleringer, som blev anvendt i flere studier i litteraturen, var, at den intuitive modelstruktur og resultaterne er lettere at kommunikere til interessenterne. Dette aspekt er en forudsætning for et vellykket resultat, men undervurderes desværre hyppigt i forskning af smitsomme sygdomme.

Diagnostiske teststrategier og overvågning

Flere studier i denne afhandling dækker forskellige aspekter af diagnostiske teststrategier, design og evaluering af overvågningsprogrammer for *S*. Dublin i kvægbesætninger. Testning til at bestemme infektionsstatus for enkeltindivider er desværre svært. Baseret på studierne i afhandlingen anbefales det, at bruge antistofmålinger på grupper af dyr til evaluering af om smittespredningen er ophørt som en del af bekæmpelsesindsatsen snarere end gentagne målinger på enkeltdyr til udpegning af potentielle smittebærere til udsætning, som hidtil anbefalet.

Bakteriologisk undersøgelse af fæcesprøver har generelt ringe diagnostisk sensitivitet i naturligt smittet kvæg og bør kun bruges til evt. bekræftelse af infektion og typning af isolater i kliniske syge, ubehandlede dyr, eller hvis det vurderes nødvendigt at forsøge at påvise mistænkte smittebærere i en gruppe af dyr, som bliver ved med at have meget høje niveauer af antistoffer. Tidligere blev det anbefalet at bruge en metode til udpegning af persistente smittebærere baseret på to eller tre høje antistofmålinger med 90 til 120 imellem. Denne metode blev evalueret i denne afhandling, og der blev ikke fundet tilstrækkeligt høj prædiktionsværdi af metoden til at udpege dyr der udskiller bakterier i gødningen i endemisk smittede besætninger. Mindre end 30 % af kvæg med en vedvarende høj antistofprofil udgjorde en risiko for at udskille bakterierne, og den højest risiko på omkring 5 % blev fundet i kvæg under 2 år gamle. Dette komplicerer brugen af test- og udsætningsstrategier som værktøj i bekæmpelsesprogrammer for *S*. Dublin. I køer over 3 år gamle var sandsynligheden for i positiv gødningsprøve under 2 % uanset hvilket antistofniveau eller hvilken antistofprofil, dyret havde.

Teststrategier til besætningsdiagnosticering baseret på antistofmålinger var generelt mere følsomme end teststrategier baseret på bakteriologisk påvisning. Den mest følsomme teststrategi rapporteret i et tidligere studium var en kombination af tankmælks-antistofmålinger og serologi af kalve mellem 4-6 måneder gamle. I det danske overvågningsprogram baseres klassificeringen af besætninger på gennemsnittet af fire på hinanden følgende tankmælks-antistofmålinger automatisk indsamlet på kvartalsbasis. Denne metode havde sammenlignelig sensitivitet og specificitet og langt lavere omkostninger. Forbedret prædiktiv værdi kan måske opnås ved risikobaserede varslingssystemer i lavrisikobesætninger med opfølgning i testpositive besætninger. Et kompromis mellem sensitivitet, specificitet og omkostninger er uundgåelig, når man vælger en prøveudtagningsstrategi til overvågning af *S*. Dublin baseret på blodprøver af ikke-mælkeleverende kvægbesætninger. De stikprøver på 8 til 10 dyr, der anvendes i de danske overvågnings-og kontrolprogrammer til forskellige formål er en følsom besætningsteststrategi. Men hvis der kun er ét dyr, der har en høj antistofreaktion, kan det være enten på grund af en meget lav prævalens, hvilket er usandsynligt i udbrudssituationer eller en falsk positiv reaktion, som der bør følges op på ved yderligere undersøgelser i besætningen. En alternativ teststrategi kan være at sætte kriteriet for en testpositiv besætningsdiagnose til to seropositive ud af 8-10 prøver fra besætningen. Det ville føre til få falskpositive besætningsklassifikationer, og vil stadig give mulighed for påvisning af de smitsomme besætninger med en rimelig følsomhed.

Bekæmpelse og udryddelse af S. Dublin

De 16 artikler, der følger med denne afhandling, har bibragt ny viden og videnskabelig dokumentation til beslutningsstøtte om metoder og tilgange, der kan bruges nationalt og regionalt, eller indeholder anbefalinger til landmænd, der ønsker at bekæmpe infektionen i deres besætninger. En artikel beskæftiger sig specifikt med bekæmpelse på nationalt plan. Anbefalingerne fra det studium blev brugt til at justere elementer i det nationale overvågningsprogram i 2006 og en mere intensiveret udryddelseskampagne blev iværksat i 2007 med fokus på den eksterne og interne smittebeskyttelse i smittede besætninger. Forslag til at kombinere disse bestræbelser med regionale flytterestriktioner og strengere regler for flytning af dyr fra smittede eller testpositive besætninger er ved at blive implementeret i programmet.

To andre studier i afhandlingen blev dedikeret en undersøgelse og demonstration af en struktureret tilgang til bekæmpelsen af *S*. Dublin i smittede kvægbesætninger samt anvendelsen af

test- og udsætningspraksis i 10 malkekvægsbesætninger, der forsøgte at udrydde infektionen fra deres kvægbesætning over en 3-årig periode. De valgte kontrolelementer var besætnings-specifikke, og de virkede for ni af de ti deltagende besætninger, der ikke købte kvæg fra testpositive besætninger. Aflivning af gentagne antistofpositive køer var kun praktisk muligt for landmænd, når forekomsten var reduceret markant i besætningerne, og når der var tegn på, at smittespredningen var ophørt blandt kalve og ungdyr.

Konklusioner og perspektiver

Den forskning, der præsenteres i denne afhandling, giver videnskabelig dokumentation for, at de nødvendige tekniske, epidemiologiske og økonomiske forudsætninger for at udrydde *S*. Dublin findes i Danmark, og hvordan det kan opnås på en effektiv måde. Danmark bør med en fhv. stor kvægsektor og høje standarder for fødevaresikkerhed, dyresundhed og dyrevelfærd være i stand til at sikre de administrative, operationelle og finansielle ressourcer, der kræves for at udrydde *S*. Dublin. De skridt, der hidtil er taget indikerer kraftigt, at det er muligt. Det er svært at se, hvordan den danske tilgang til udryddelse af *S*. Dublin skulle blive forhindret af andre socioøkologiske faktorer end eventuelt nogle modstridende opfattelser, holdninger og adfærd blandt nogle modvillige landmænd og lokale dyrlæger og kvægrådgivere. I denne afhandling blev det beskrevet, hvordan sådanne opfattelser og adfærd kan påvirkes ikke kun af lovgivningen, men også gennem ejeres eller driftslederes deltagelse i feltprojekter for smittede besætninger, der har behov for støtte til at gennemføre den krævede bekæmpelsesindsats. Mange af de metoder, der anvendes i Danmark, og er beskrevet her kunne med fordel implementeres i andre lande med mælke- og oksekødsproduktion og problemer med *S*. Dublin infektioner.

Det anbefales, at fremtidige studier fokuseres på at undersøge diffuse smitteveje, der kan hindre bekæmpelsesprogrammets succes, såsom betydningen af transport og spredning af forurenet gødning/gylle, store flokke af trækfugle, adgang til og forurening af overfladevand. Desuden anbefales det, at forekomst og potentielle kilder til andre salmonellaserotyper i de danske kvægbesætninger undersøges. En potentiel stigning i nye salmonellaserotyper kan føre til nye sygdomsmønstre i smittede besætninger og krydsreaktioner i antistoftestene i overvågnings-programmet. Endelig kan det være værd at vurdere alternative bekæmpelsesscenarier i bestemte besætninger med usædvanlige betingelser, der fører til persisterende infektion, f.eks. meget store besætninger, økologiske besætninger, besætninger med mange ejendomme eller bygninger mv., da disse kan have særlige udfordringer med at bekæmpe *S*. Dublin.

List of accompanying papers

This thesis is based on 16 original manuscripts, which are published in peer-reviewed international journals. Roman numbers I to XVI are used to refer to the papers in Chapters 1 to 8.

Review of pathogenesis and diagnostic methods (Chapter 2)

I. Nielsen, L.R. **Review of pathogenesis and diagnostic methods of immediate relevance for epidemiology and control of *Salmonella* Dublin in cattle**
Veterinary Microbiology (2013), 16, 1-9.

Occurrence (Chapter 3)

II. Nielsen, L.R.
Within-herd *Salmonella* Dublin prevalence in endemically infected dairy herds
Epidemiology & Infection (2013), Online: http://dx.doi.org/10.1017/S0950268812003007

III. Nielsen, L.R., Borne, B. van den and Schaik, G. van.
***Salmonella* Dublin infection in young dairy calves: Transmission parameters estimated from field data and an SIR-model**
Preventive Veterinary Medicine (2007), 79, 46–58

IV. Nielsen, L.R., Baggesen, D.L., Aabo, S., Moos, M.K. and Rattenborg, E.
Prevalence and risk factors for *Salmonella* in veal calves at Danish cattle abattoirs
Epidemiology & Infection (2011), 139, 1075–1080.

V. Nielsen, T. D., Nielsen, L. R. and Toft, N.
Bayesian estimation of true between-herd and within-herd prevalence of *Salmonella* in Danish veal calves
Preventive Veterinary Medicine (2011), 100, 155–162.

Risk factors (Chapter 4)

VI. Nielsen, L.R., Warnick. L.D. and Greiner, M.
Risk factors for changing classification status in the Danish surveillance program for *Salmonella* in dairy herds
Journal of Dairy Science (2007), 90, 2815–2825.

VII. Nielsen, L.R., Dohoo, I.
Survival analysis of factors affecting incidence risk of *Salmonella* Dublin in Danish dairy herds during a 7-year surveillance period
Preventive Veterinary Medicine (2012), 107, 160-169.

VIII. Nielsen, L.R., Dohoo, I. **Time-to-event analysis of predictors for recovery from *Salmonella* Dublin infection in dairy herds**
Preventive Veterinary Medicine (2013), 110, 370-378.

Infection dynamics (Chapter 5)

IX. Nielsen, L.R., Kudahl, A.B. and Østergaard, S.
Age-structured dynamic, stochastic and mechanistic simulation model of *Salmonella* Dublin infection within dairy herds
Preventive Veterinary Medicine (2012), 105, 59-74.

X. Nielsen, T.D., Kudahl, A.B., Østergaard, S. and Nielsen, L. R.
Gross margin losses due to *Salmonella* Dublin infection in Danish dairy cattle herds estimated by simulation modelling
Preventive Veterinary Medicine (2013), 111, 51-62.

XI. Jordan, D., Nielsen, L.R. and Warnick, L.D.
Modelling a national program for the control of food-borne pathogens in livestock: the case of *Salmonella* Dublin in the Danish cattle industry
Epidemiology & Infection (2008), 136, 1521–1536.

Diagnostic test-strategies and surveillance (Chapter 6)

XII. Nielsen, L.R.
***Salmonella* Dublin faecal excretion probabilities in cattle with different temporal antibody profiles in 14 endemically infected dairy herds**
Epidemiology & Infection (2013), Online: http://dx.doi.org/10.1017/S0950268812002579

XIII. Lomborg, S., Agerholm, J., Jensen, A. and Nielsen, L.
Effects of experimental immunosuppression in cattle with persistently high antibody levels to *Salmonella* Dublin lipopolysaccharide O-antigens
BMC Veterinary Research (2007), 3, 17.

XIV. Warnick, L.D., Nielsen, L.R., Nielsen, J. and Greiner, M.
Simulation model estimates of test accuracy and predictive values for the Danish *Salmonella* surveillance program in dairy herds
Preventive Veterinary Medicine (2006), 77, 284-303.

Controlling *S.* Dublin in cattle herds (Chapter 7)

XV. Nielsen, L.R. and Nielsen, S.S.
A structured approach to control of *Salmonella* Dublin in 10 Danish dairy herds based on risk scoring and test-and-manage procedures
Food Research International (2012), 45, 1158-1165.

XVI. Nielsen, L.R. and Dohoo, I.R.
Culling decisions of dairy farmers during a 3-year *Salmonella* control study
Preventive Veterinary Medicine (2011) 100, 29-37.

Chapter 1
Introduction

Historical background

Salmonella enterica subsp. *enterica* serovar Dublin (*S.* Dublin) is a bacterial infection that has received a lot of attention in the Danish cattle industry and the Danish Veterinary and Food Administration since the 1990s. There are several reasons for this:

- *S.* Dublin is **host-adapted to cattle**. Thus, it is mainly found in bovines, but also lead to disease in humans (Jones et al., 2008), ovines (Uzzau et al., 2000) and occasionally in other species (Dietz et al., 2006).
- ***S.* Dublin is a zoonosis** that leads to occasional severe invasive infections with high case fatalities in humans (Mandal and Brennand, 1988; Helms et al., 2003; Jones et al., 2008) after consumption of contaminated beef, intake of contaminated unpasteurised milk products (Maguire et al., 1992) or direct contact with infected cattle (Mateus et al., 2008). The annual number of recorded human cases in Denmark varied between 0 and 49 from 1980 to 2011 (Lester et al., 1995; Anonymous, 2011).
- *S.* Dublin has been **common in the Danish cattle population** for more than 100 years (Jensen, 1891; Nielsen, 2003). Observational studies quantified the occurrence in the 90s: a national study found that 73% of dairy herds were repeatedly antibody negative and 11% were repeatedly antibody positive in three bulk-tank milk test rounds between 1994 and 1996. The herd level prevalence of antibody positive dairy herds appeared to increase from 17% to 21% in the same period (Wedderkopp, 1996).
- *S.* Dublin infection has been associated with a **compromised animal health and welfare** in infected herds (Nielsen et al., 2010). In a large field study of 223 outbreak herds in the North West of England, it was found that *S.* Dublin led to adult dysentery in 18%, abortions in 13% and calf hood disease such as diarrhoea and pneumonia in 86% of the outbreak herds. The associated mortality was 47% in 60 cows with dysentery, whereas none of the aborting cows died. In total, 33% of the calves (n=6,239) in these herds became clinically ill and half of the diseased calves died. However, there was much variation in the morbidity and mortality between the herds (Richardson and Watson, 1971).
- *S.* Dublin infection has also been **associated with economic losses** (Nielsen et al., 2012a). One study found the cost of an *S.* Dublin-outbreak in a calf rearing unit with 214 animals to be £4,691 or £25.36 per survivor in 1982-figures. This was a substantial proportion of the gross margin gained from animals sent to slaughter. The main causes for the losses in that study were an increased calf mortality and veterinary expenses (Peters, 1985). Another study attempted to quantify the production losses ascribed to *S.* Dublin in 40 dairy farms in the Netherlands. Losses related to abortions were mainly important due to the other effects related to the abortion such as culling patterns, loss of milk production and prolonged calving intervals. Calf mortality and veterinary expenses were also important losses. The average total loss was 5,000 Dutch guilders (or 55 guilders per cow), but this could go up to 18,000 Dutch guilders in 1997-figures in the worst cases. This was approximately 4.5 % of the net return to labour and management (Visser et al., 1997). One Dutch guilder corresponds to approximately 0.45 euro.

Moreover, there has been an increasing focus on the need to control *Salmonella* infections in livestock in the European Union to live up to the microbiological criteria in food products (European Commission, 2005). In Denmark, the poultry and pig industries have had *Salmonella* surveillance and control programmes in place since the mid 1990s. Therefore, there has been an increasing political pressure for similar initiatives in the cattle industry.

Literature suggests that *S*. Dublin has a tendency to produce a persistent infection stage in cattle often referred to as carriers or latent carriers (Richardson, 1973; House et al., 1993), which supposedly influences transmission patterns within and between cattle herds. Thus, *S*. Dublin frequently presents with epidemiology that differs from e.g. *S*. Typhimurium, the second most commonly isolated serotype of *Salmonella* in cattle (Lawson et al., 1974; Wray and Snoyenbos, 1985).

Consequently, a surveillance programme in all of the Danish cattle herds was developed and initiated in October 2002 to monitor the prevalence over time and provide farmers with information that allowed them to protect their herd from an introduction of *S*. Dublin through a purchase or other types of contact with infected herds (Anonymous, 2004).

Three PhD projects, two Danish and one Dutch, developed and investigated diagnostic test accuracies and predictive values of the available laboratory tests for a diagnosis of the *S*. Dublin infection at animal and herd level, i.e. conventional bacteriological culture methods and the more newly developed serological diagnostic methods (ELISA) on serum and milk samples (Wedderkopp, 2000; Nielsen, 2003; Veling, 2004). However, for the surveillance programme to be effective and successful, more knowledge was required regarding the epidemiology of *S*. Dublin in Denmark. Furthermore, the evaluation of optimal test-strategies and control scenarios to improve efficiency and to ensure success in a control programme were requested.

Aim

The aim of this thesis was to produce new evidence-based knowledge about *S*. Dublin epidemiology including the occurrence and infection dynamics of relevance for the design and the evaluation of surveillance and control programmes for *S*. Dublin in cattle populations by presenting and discussing the results of 16 epidemiological studies performed in Denmark between 2000 and 2012. Furthermore, this thesis includes a review paper on the pathogenesis and diagnosis of *S*. Dublin that contains the knowledge needed to understand and interpret the results presented.

Hypotheses

The overall hypothesis pursued in this thesis is that cost-effective control of *S*. Dublin in cattle can be achieved through targeted management actions directed against herd specific risk factors for within-herd and between-herd transmission of *S*. Dublin. The control progress can be monitored and supported by diagnostic test-strategies at animal group and herd level, and surveillance at regional and national level.

This hypothesis was investigated through studies within the following main topics:

A. **Occurrence** including an estimation of prevalence, incidence and duration of infection.
B. **Risk factors** in dairy and non-dairy herds based on register data from the Danish Cattle Database and data from field studies.
C. **Infection dynamics** at herd and national level, an estimation of animal health and the economic consequences of an infection in dairy herds.
D. **Diagnostic test-strategies and surveillance** at animal and herd level in dairy and non-dairy herds including an evaluation of the performance of the Danish surveillance programme in dairy herds.
E. **Control and eradication** of *S.* Dublin in cattle herds by the use of systematic approaches to target control actions combined with test-strategies to assess the progress.

The following specific hypotheses were investigated in the thesis:

(1) The within-herd prevalence of *S*. Dublin in endemically infected dairy herds varies between age-groups and between the seasons.
(2) The variation of within-herd prevalence of *S*. Dublin is related to the dynamics of the infection, the herd immunity and the dynamics of the population at risk.
(3) The prevalence of *S*. Dublin in veal calves is higher than in dairy herds due to a continued purchase and mixing of calves from different suppliers including infected herds.
(4) *S*. Dublin prevalence estimates obtained without taking the accuracy of the diagnostic tests into account are often underestimated, because of lack of sensitivity in the available test methods.
(5) Risk factors for an introduction of *S.* Dublin to dairy herds include both direct infection routes such as the purchase of cattle from infected herds and more diffuse or indirect infection routes from infected neighbouring herds.
(6) The risk of an introduction or re-introduction of *S.* Dublin is higher for a period after a herd has been infected and becomes test-negative than it is for herds with no prior history of infection.
(7) Internal biosecurity factors are important for the persistence of an *S.* Dublin infection, whereas external factors may play a minor role for the duration of the infection or the tendency for the infection to persist in an infected cattle herd.
(8) Within-herd *S.* Dublin infection dynamics depend on age-group population dynamics, herd size, hygiene and other herd specific management practices.
(9) *S*. Dublin affects animal health and welfare and leads to significant economic losses in dairy herds upon an introduction and spread of the infection within the herds.
(10) The magnitude of the economic losses associated with *S*. Dublin in dairy herds depends on the herd size, hygiene and management.
(11) Cattle with persistently high *S*. Dublin antibody levels will excrete bacteria in faeces more frequently than cattle with persistently low or fluctuating antibody levels and therefore pose a significant risk of spreading the infection in the herd.
(12) Immunosuppression of cattle with persistently high *S*. Dublin antibody levels will reactivate latent infections and lead to the shedding of bacteria in faeces and milk.

(13) The classification system in the Danish *S*. Dublin surveillance programme leads to very few false negative herd classifications, but some false positive classifications due to antibodies remaining in bulk-tank milk after dairy herds recover from the infection.
(14) The sample sizes of 8 or 10 animals used in the Danish surveillance and control programmes of non-dairy herds provide sensitive herd diagnoses and are suitable for the purpose of the programme: to follow the national prevalence of infection in non-dairy herds over time.
(15) Strict internal and external biosecurity measures are essential for success in a national control and eradication programme for *S*. Dublin.
(16) Concomitant participation in other disease control programmes such as a paratuberculosis control programme is beneficial when controlling *S*. Dublin in dairy herds.
(17) Effective control of *S*. Dublin in dairy herds can be reached through herd specific targeted management practices based on risk scoring and test-and-manage procedures.
(18) The culling decisions of farmers with *S*. Dublin infected dairy herds who participate in a control programme depend on the number and proportions of cattle with high-risk categorisations in addition to other factors of importance for these culling decisions.

Outline of the thesis

The work presented in this thesis was performed as 16 studies in continuation and further development of the knowledge and experiences were presented in the PhD thesis "*Salmonella* Dublin in dairy cattle: Use of diagnostic tests for investigation of risk factors and infection dynamics" from The Royal Veterinary and Agricultural University (Nielsen, 2003) and other literature. A general introduction to *S*. Dublin including a state of the art pathogenesis and a diagnosis of *S*. Dublin in cattle of immediate relevance for control of the infection is needed to understand and discuss the results of the studies in the rest of the thesis. This is therefore provided in Chapter 2. That chapter constitutes a review paper that has been accepted for publication after peer-review (Paper I). The results of my own work are presented and discussed in Chapters 3 to 7 based on the studies in the accompanying papers and in relation to the relevant literature within the main topics mentioned above. Conclusions and perspectives for future control, eradication and prevention of *S.* Dublin in cattle populations are provided in Chapter 8.

Chapter 2
Review of pathogenesis and diagnostic methods of immediate relevance for epidemiology and control of *Salmonella* Dublin in cattle (Paper I)

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Abstract
Salmonella enterica subsp. *enterica* serovar Dublin (*S.* Dublin) receives increasing attention in cattle production. It is host-adapted to cattle, and leads to unacceptable levels of morbidity, mortality and production losses in both newly and persistently infected herds. Cattle health promoting institutions in several countries are currently constructing active surveillance programmes or voluntary certification programmes, and encourage control and eradication of *S*. Dublin infected cattle herds. There is a need to understand the underlying pathogenesis of the infection at both animal and herd level to design successful programmes. Furthermore, knowledge about and access to diagnostic tests for use in practice including information about test accuracy and interpretation of available diagnostic test methods are requested.

The aim is to synthesise the abundant literature on elements of pathogenesis and diagnosis of immediate relevance for epidemiology and control of *S*. Dublin at animal and herd level. Relatively few in vivo studies on *S*. Dublin pathogenesis in cattle included more than a few animals and often showed varying result. It makes it difficult to draw conclusions about mechanisms that affect dissemination in cattle and that might be targets for control methods directed towards improving resistance against the bacteria, e.g. new vaccines. It is recommended to perform larger studies to elucidate dose-response relationships and age- and genetic effects of immunity. Furthermore, it is recommended to attempt to develop faster and more sensitive methods for detection of *S*. Dublin for diagnosis of infectious animals.

Introduction
Salmonella enterica subsp. *enterica* serovar Dublin (*S*. Dublin) belongs to the genus *Salmonella* in the family Enterobactericeae. It is one of many *Salmonella* serovars and is a Gram negative, oxidase-negative and rod-shaped bacterium. The bacteria are usually not particularly resistant to disinfectants, direct sunlight and antibiotics, even though multidrug-resistant strains have been isolated from beef and dairy sources (Davis et al., 2007). However, it can survive for months in organic matter such as stored slurry, cattle manure and soil (Taylor and Burrows, 1971) and for years in dried-in faecal matter (Plym-Forshell and Ekesbo, 1996). The survival of *S*. Dublin in slurry depends on temperature, pH, other microflora and treatment and slurry storage conditions (Jones, 1976; Jones et al., 1977). The bacteria can multiply outside the host under warm and moist circumstances (Wray and Davies, 2000). Even though environmental contamination should be considered, the present review focuses on the host and agent specific factors that are relevant to the epidemiology and control of *S*. Dublin.

Pathogenesis
The purpose of this section is to review studies on pathogenesis of immediate relevance for diagnosis, epidemiology and control of *S*. Dublin. Hence, it only touches briefly on the abundant

molecular biological research that has been performed to improve understanding of cellular mechanisms in the pathogenesis of *S*. Dublin and other *Salmonella* serotypes and focuses mainly on uptake, dissemination, infectiousness, immune responses and clinical signs in cattle. The pathogenesis of *S*. Dublin in the host depends on factors such as infection dose, passive transfer of specific immunoglobulins, immunity developed during previous infections, age at infection and physiological state of the host. Current knowledge on the pathogenesis of *S*. Dublin has been derived from a mix of experimental studies and epidemiological field studies as summarised below.

Uptake of bacteria

S. Dublin most commonly infects the host after direct oral uptake of contaminated food (including milk) and water or from contaminated environment, pen mates, calf or dam (Nazer and Osborne, 1977; Hardman et al., 1991). Less common entrance routes include airways and conjunctiva (Nazer and Osborne, 1977). These entrance routes may be important under special circumstances, e.g. when pressure washing is used and live animals are present in the barn, which allows contaminated steam and aerosols to be spread and gain access to the airways or conjunctiva. *S*. Dublin is also able to infect the foetus in-utero. However, in-utero infection will often lead to abortion or still born calves (Hinton, 1974). Introduction through the mammary glands is not a common route of infection, even though one study showed that persistent *S.* Dublin carriers with chronic mastitis and systemic reactions could be artificially created by experimental inoculation of low numbers of *S*. Dublin bacteria through the teat canal followed by chemically induced immunosuppression (Spier et al., 1991).

Infectious dose

The infectious dose of *S*. Dublin may be strain dependent (Wallis et al., 1995), but infectious doses of more than 10^6 cell forming units (CFU) per os usually lead to clinical signs and/or shedding of bacteria in calves between 0 and 6 months of age. The higher the infection dose, the more consistently shedding and clinical signs can be reproduced, and the severity of clinical signs and pathological changes vary with the age of the infected animal (Nazer and Osborne, 1977; Robertsson, 1984; Segall and Lindberg, 1991; Steinbach et al., 1993). Pre-weaned calves (usually below 6 to 8 weeks old) are highly susceptible (Nazer and Osborne, 1977; Segall and Lindberg, 1991). However, the mentioned studies were performed in experimental settings. Common farming conditions may result in completely different environments with continuous or intermittent exposures to smaller doses of bacteria, which can still lead to infection and excretion of bacteria, but with fewer and milder clinical signs (Wray and Sojka, 1981).

There are only few studies on *S*. Dublin infections in older cattle and these give varying results. Peroral doses of 10^{10} or 10^{11} CFU were found to lead to variable responses from no clinical signs to severe illness with dysentery, pyrexia and abortions in nine pregnant heifers (Hall and Jones, 1979). Intravenous inoculation of 10^9-10^{10} CFU given to heifers aged 27 to 44 months lead to severe illness in the infected heifers (Hall and Jones, 1977). Naturally infected cows aborting under field conditions often have few or no other clinical signs than transient pyrexia, which may easily be overlooked under normal farm circumstances (Richardson and Watson, 1971; Hinton, 1974). The differences between study results can probably be explained by lower infection doses under natural farm conditions and the fact that natural infection enters the host via the mouth and gastrointestinal canal and not intravenously.

Dissemination in the host

After colonisation of the gut, *Salmonella* bacteria can adhere to and invade columnar enterocytes mainly in the terminal jejuna and ileal mucosas, and then pass through to the lymphatic tissues beneath. Here they enter macrophages that are drained to the local lymph nodes. This is an important barrier for further dissemination. If they pass this barrier, the bacteria reach the lymph and blood (bacteraemia) and the internal organs containing the mononuclear phagocyte system (e.g. in the spleen and liver), and the tonsils, lymph nodes and lungs, while surviving and replicating inside macrophages (Segall and Lindberg, 1991). The differences in clinical signs and epidemic sizes in cattle herds might be explained to some extend by differences in virulence of *S*. Dublin strains. Some strains are better at penetrating the intestinal wall and the barriers into the lymphatic system than others, and some strains have a better intracellular survival than other strains (Watson et al., 1995; Wallis et al., 1995). Oral inoculation with *S*. Dublin strains in 4-week-old calves showed that invasion of the enterocytes in intestinal mucosa in the ileum, colon and caecum was evident at 90 minutes, 3 days and 6 days after the oral challenge indicating that the invasion of the intestinal wall can be rapid and may continue for days (Wallis et al., 1995). Some strains of *S*. Dublin has been found to be highly invasive as well as histotoxic leading to a higher degree of destruction of the enterocytes than other serotypes (Bolton et al., 1999). At investigated systemic sites (e.g. liver, spleen, hepatic and bronchial lymph nodes) the concentrations of bacteria were generally lower than at the investigated intestinal sites. Differences in presence at the systemic sites were evident between different *S*. Dublin strains at 6 days post-infection indicating potential reasons for strain differences in virulence and persistence. Diarrhoea was present in most of the challenged calves, whereas clinical signs of systemic disease (i.e. pyrexia and anorexia) and death were mainly seen in calves challenged with plasmid-carrying wild-type strains (Wallis et al., 1995).

Infection stages

There are ample suggestions in the literature that *S*. Dublin can lead to a latent carrier stage that might be important for persistence of infection in infected herds, e.g. *S*. Dublin has been found in the internal organs of non-shedding cattle (Smith et al., 1989; Spier et al., 1991; House et al., 1993; Lomborg et al., 2007 **Paper XIII**). When investigating and describing the epidemiology of *S*. Dublin it is useful to differentiate between acute and persistent clinical and subclinical (asymptomatic) infection stages that cattle can experience upon becoming infected (Table 2.1) (Richardson, 1973; Robertsson, 1984; Rings, 1985; Wray and Davies, 2000; Loeb et al., 2006).

Infectiousness

S. Dublin bacteria may be shed trough milk, urine, saliva, vaginal discharge and faeces. Duration of excretion and amounts of bacteria excreted by infected cattle are highly variable. Since faeces contains the highest number of bacteria and is produced in largest quantities, it is the most important vehicle of transmission of *S*. Dublin. Therefore, faecal samples are frequently used to assess whether cattle are excreting bacteria (Veling, 2004; Davison et al., 2005).

Not all infected cattle excrete bacteria, but when they do, faecal shedding of *Salmonella* bacteria can start as early as 12 to 48 hours after uptake of the bacteria, and shedding can last several months (House et al., 1993; Veling, 2004). The average duration has been reported to be 15 to 17 days in calves with clinical signs and shorter in older animals and animals with subclinical infections (Robertsson, 1984; Nielsen et al., 2007 **Paper III**). Occassionally, shedding may continue

for months or years, usually intermittently. In such cases the animals are considered persistently infected carriers (House et al., 1993; Veling, 2004; Nielsen et al., 2004a). The terminology used in the literature for different types of persistently infected stages is confusing. Most agree that 'active carriers' are animals that frequently or continuously excrete bacteria, and that 'passive carriers' can transfer the bacteria through the gut without becoming clinically affected. However, the term 'latent carrier' in some instances refers to a non-excreting infected animal that may or may not become reactivated in the future. In other instances, it is referred to as an animal that excretes bacteria intermittently and therefore should be considered a moderately high risk animal regarding transmission of infection within and between herds (Richardson, 1973; House et al., 1993; Nielsen et al., 2004a). Regardless of the definition, persistent carriers need to be taken into account in control of *S*. Dublin and in test-strategies to support control efforts.
Even though there are several coherent studies on duration of infection, the amounts of bacteria excreted by each infectious animal vary vastly. There are reports that persistently infected cows can excrete moderate numbers (between 10^4 and 10^5 CFU/g faeces), whereas other cows excreted low numbers (10^1 CFU/g to 10^4 CFU/g faeces) for a period of at least 30 months (Sojka et al., 1974). In a Danish field study of cows in two endemically infected herds, semi-quantitative evaluations of *S*. Dublin concentrations in faecal samples ranged from very low or low (0.2 to 10^4 CFU/g) in cows (unpublished data). For comparison, a wide range of concentrations (0.2 to 10^8 CFU/g) was found in calves during a clinical outbreak of *S*. Dublin in a dairy herd (unpublished data). These results were supported by studies of slaughter cattle in which it was found that the majority of test positive samples from cattle at slaughter had <10 CFU/g faeces (Nielsen et al., 2011 **Paper IV**). Hence, the general pattern is that subclinically or persistently infected cattle shed low numbers of *S*. Dublin, whereas clinically ill or acutely infected animals may excrete high numbers in faeces.

Immune responses

The cellular and humoral immune components of the immune system act in combination to combat *Salmonella* infections. The innate immune system consists of inflammatory cells such as macrophages, polymorphonuclear leukocytes, neutrophils, natural killer cells and their secreted cytokines, and it constitutes the first line of defence against invading *Salmonella* bacteria. This non-specific immune system activates the adaptive part of the immune system. The IgM and IgG titres begin to increase approximately one week and two weeks, respectively, after inoculation in calves infected at the age of 6 to 7 weeks (Robertsson, 1984). The maximum titre of IgG is reached between 6 and 11 weeks after inoculation. It then gradually decreases and reach baseline levels around 14 to 20 weeks after inoculation (i.e. between 2 and 3 months after peak IgG titre) unless re-infection occurs (Robertsson, 1984; Smith et al., 1989). Older cattle have faster IgG responses than calves. In calves infected below the age of 11 weeks production of specific antibodies is poorer and slower than in older cattle, and it may take several months to reach measureable levels of circulating antibodies (Da Roden et al., 1992).

Table 2.1 Clinical characteristics, duration and infectiousness of different infection stages of *S*. Dublin upon oral uptake in susceptible cattle

Infection stages and characteristics of infection stages	Duration	Shedding of bacteria
Acute infections		
Peracute		
Death after a short period of bacteraemia followed by endotoxic shock. No other clinical signs, or similar to the acute infection stage. Most common in naïve herds.	1-2 days	Animals often die before they start to excrete bacteria
Acute		
Local enteric infection or systemic infection with transient bacteraemia. In calves clinical signs are sometimes seen and include: hyperthermia, depression, loss of appetite, pneumonia, bloody/watery diarrhoea, arthritis and in rare cases nervous symptoms. In adult cattle it is more common to see bloody/watery diarrhoea, hyperthermia, depression, abortion, decreased milk production and loss of appetite.	1-3 weeks, may extend to 5-9 weeks	Animals may shed large amounts (from 1 to 10^8 CFU/g) in faeces, urine, vaginal discharge, and milk, continuously or intermittently
Persistent infections		
Chronic infection		
Follows acute infection usually in calves older than 6 to 8 weeks. Clinical signs include failure to thrive, bloody/loose stool, shedding of intestinal casts, slightly elevated temperature, scruffy hair coat and growth retardation. Lameness is common due to arthritis or osteomyelitis. Ischemic necrosis of the skin on ears, tail or distal limbs may occur.	Months	The animal may or may not be shedding bacteria
Passive carrier		
Subclinical. Passive carrier of bacteria in the lumen of the gut, no invasion of intestinal epithelium	Weeks to months	Shedding periodically in faeces until removed from source
Latent carrier		
Subclinical. Latent carriers of bacteria in lymphoid tissues. May periodically become reactivated from stress or other unknown causes.	Months to years	Not shedding unless reactivated and then usually only sheds low amounts of bacteria (1-10^4 CFU/g)
Active carrier		
Subclinical. Active carriers (by some referred to as super shedders). May carry the bacteria in both the lumen of the gut, gut-associated tissues, lymphoid system and internal organs.	Months	Intermittent or continuous shedding at similar levels as acutely infected

Previous infection can have some protective effect, which in practice means that re-infected animals typically show fewer clinical signs and excrete lower numbers of bacteria. However, the number of bacteria in the lymph nodes might be higher in re-infected calves than in naïve animals (Steinbach et al., 1996). This may be related to cell-mediated immunity which is more protective against an *S*. Dublin infection than humoral immunity (Robertsson, 1984). The cell-mediated immune response is not necessarily correlated to the level of circulating antibodies. Calves with well-developed cell-mediated immunity had fewer and milder symptoms upon re-infection, and duration of bacterial excretion was shorter than in control calves. Calves with high levels of immunoglobulins originating from passive transfer did not have the same degree of protection (Chaturvedi and Sharma, 1981). *S*. Dublin has been shown to survive in the mammary gland for more than a year despite high levels of antibodies directed against *S*. Dublin (Spier et al., 1991). This indicates that the humoral immunity is not sufficient to clear the infection. Hence, antibodies can be useful for diagnostics of exposure to *S*. Dublin bacteria, but cannot be used for prognostic purposes. Observational studies investigating the effect of herd level immunity on the spread and clinical expression of an *S*. Dublin infection, and whether such herd immunity is correlated to the number of antibody positive animals in the herd are lacking.

Host adaptation

S. Dublin is host adapted to cattle. Different hypotheses have been suggested as to why this is, including speculations about differences in evolution of *Salmonella* pathogenicity islands (Bispham et al., 2001). So far there is no general agreement about the underlying mechanisms of host adaptation. Host specificity is most likely caused by a unique set of mechanisms for each of the host adapted or host restricted *Salmonella* serotypes rather than one common explanation for the host specificity phenomenon (Uzzau et al., 2000). In relation to control of the infection the actual mechanism of host adaptation might be less important, but the fact that *S*. Dublin is host adapted makes it feasible for the cattle health institutions to initiate effective control programmes without involvement of other sectors. This makes effective control feasible.

Host factors affecting pathogenesis

Genetic host factors play a role in *S*. Dublin pathogenesis. Expression of a protein in the host encoded by the gene *Slc11a1* has been found to be an important determinant of pro-inflammatory cytokine expression, which affects the level of invasion, survival in macrophages and development of clinical symptoms in relation to *S*. Typhimurium infection in mice (Valdez et al., 2008), but the role of the gene in relation to an *S*. Dublin infection in cattle is not known. Furthermore, an *Lps* locus in the host seems to regulate the ability to respond to bacterial surface lipopolysaccharides (LPS) (Scherer and Miller, 2001). Differences in expression of such genes may explain some of the differences in susceptibility between individual animals (Wigley, 2004).

The physiological state of the host is important for progression of infection upon uptake. In weaned calves and adult animals, the physiological states of the rumen and the rest of the gastrointestinal tract are important for the multiplication and colonisation of the gut, which precedes invasion of the intestinal epithelium. Volatile fatty acids in the rumen and the low pH (<4.8) in the abomasum inhibits multiplication of *Salmonella* bacteria (Mattila et al., 1988). Peristalsis and competing microflora of the rumen and the small intestines prevent adhesion to the epithelial cells. It takes either sufficiently high infection doses or a disruption of the normal function of the gastrointestinal tract to allow *Salmonella* bacteria to multiply, colonize and invade

the epithelium in the small intestine. Such disruption may occur during starvation, deprivation of water, transportation, other diseases, sudden changes in feeding routines, poor quality feed, severe weather conditions and antibiotic treatment. Moreover, concomitant infections with e.g. Bovine Virus Diarrhoea virus (BVDv) and *Fasciola hepatica* can aggravate the *S*. Dublin infections, make the host more susceptible to becoming infected or prevent the host from clearing S. Dublin infection (Aitken et al., 1981; Wray and Roeder, 1987).

Bacterial factors affecting pathogenesis and diagnosis

Most experimental studies of *Salmonella* pathogenesis have been performed in chickens, rats or mice which are not the most ideal species to use for inference drawing on the mechanisms of the *S*. Dublin pathogenesis in cattle. General conclusions about *Salmonella* pathogenesis are often based on studies of other serotypes than *S*. Dublin. *Salmonella* virulence plasmid genes (*spv* genes) are considered important for the pathogenesis of *S*. Dublin. However, bacterial *spv* genes may only be needed for *S*. Dublin to produce systemic disease, but do not appear to be necessary for enteric disease to occur in cattle (Wallis et al., 1995). The ability to multiply and survive intracellularly is important for the tendency of *S*. Dublin to produce prolonged carrier states (Brackelsberg et al., 1997).

Outer membrane LPS that present O-antigens to the environment of the bacteria are important for the pathogenesis of *S*. Dublin because they contain the endotoxin 'lipid A' that is released upon bacterial death. This component is a potent toxin for host cells and causes release of cytokines from monocytes and macrophages, e.g. interferon, tumour necrosis factor, colony-stimulating factor and interleukin 1. They contribute to vascular damage and thrombosis and play an important role in the tissue damage leading to hyperthermia, disseminated intravascular coagulation, circulatory collapse characteristic for endotoxic shock during *Salmonella* infections (Rycroft, 2000). Immunoglobulins from the host are directed against the O-antigens and the response is to some extend serotype specific, because different serotypes present different O-antigens on the bacterial surface. However, some *Salmonella* serotypes have common O-antigens, so cross-immunity may occur upon infection with such serotypes. For instance, cattle infected with *S*. Dublin usually produce immunoglobulins directed against LPS O-antigens 1, 9 and 12. *S*. Typhimurium may present O-antigens 1, 4, 5 and 12, so the two serotypes have O1 and O12 in common which may lead to both cross-immunity and cross-reactions in serological tests based on these antigens (Konrad et al., 1994).

Diagnosis

According to the OIE Resolution No. XXIX from 2003 diagnoses of infectious diseases should be made with a specific purpose in mind. The resolution establishes that 'fitness for purpose' should be used as a criterion for validation of diagnostic tests. The purpose might be to demonstrate freedom from disease in a unit of animals (e.g. herd, region, country), to confirm diagnosis of clinical cases or to estimate the prevalence. The diagnosis can be important for prognosis and decision-making about animals, e.g. isolation or culling. However, when performing diagnostic procedures for control and prevention of *S*. Dublin, one needs to accept 'probability diagnoses', because perfect tests for *S*. Dublin do not exist. First and foremost, it is important to establish a target condition that the diagnostic test should detect, e.g. infected, infectious or diseased animals.

Clinical and pathological signs are usually too unspecific to diagnose *Salmonella* infections with certainty. Diagnostic tests can in principle be anything that increases our knowledge about the condition in question, but currently the most commonly used diagnostic tests for *S*. Dublin infections include clinical examinations, cultivation of bacteria and measurements of antibodies directed against *S*. Dublin. These are all imperfect tests. In some instances, improved accuracy can be reached by combining diagnostic tests, e.g. clinical examinations may be helpful in increasing the sensitivity (Se) of the bacteriological detection method for confirmation of clinical suspicion by selecting animals most likely to excrete bacteria (i.e. currently or recently ill animals that have not been treated with antibiotics) (Veling, 2004).

Detection of bacteria

Detection of bacteria in faeces, organ tissues, fluids or environmental samples can be done by conventional bacteriological culture methods. These methods have the advantage of being able to identify the type of *Salmonella* in question, which is useful in tracing of infections, e.g. in outbreak investigations. The disadvantage of these methods is low Se. Newer techniques are based on detection of genetic material from the bacteria, i.e. Polymerase Chain Reaction (PCR)-techniques. These are generally thought to be more sensitive, but have the disadvantage that subsequent typing is not always possible. If the detected bacteria cannot be cultured, it is not possible to determine the serotype. This section reviews studies that have evaluated the methods currently in use or being evaluated for detection of *S*. Dublin bacteria in Denmark.

Conventional bacteriological culture

Bacteriological cultivation is based on a stepwise procedure aiming at isolating live bacteria in the sample. These include pre-enrichment, selective enrichment, plating and confirmation. The bacteria must be able to grow in the enrichment steps for the test to be positive. If the concentration of bacteria in the sample is >100 CFU/g even small sample volumes such as rectal swabs have high Se (60-100%) (Richardson and Fawcett, 1973). As discussed above this situation is mainly relevant for acutely infected or clinically ill animal. In principle, the method should be able to detect as little as one CFU in a sample. However, this is not always the case. The Se of faecal culture for detection of infected animals (e.g. potential shedders) was estimated in a latent class analysis of 4,531 paired faecal cultures and antibody measurements directed against *S*. Dublin antigens in cattle from 29 Danish dairy herds. The Se was very low, i.e. the 95% confidence interval ranged between 0% and 25% with the most likely value being 8-10% (Nielsen et al., 2004b). However, this was based on a sampling procedure in which 5 g from each of five individual samples were mixed into a pool of 25 g for initial culture. The individual samples were only analysed for bacteria if the pool was positive. This procedure reduce the relative Se for animal level diagnosis to approximately half that of the individual sample Se, so the most likely average Se of individual faecal culture for detection of infected animals among cattle not showing clinical signs is 16-20%. In practice, one reason for poor Se of faecal culture tests may be intermittent shedding in infected cattle or low concentrations excreted by subclinically or re-infected cattle (House et al., 1993; Steinbach et al., 1996; Nielsen et al., 2004a; Nielsen et al., 2007 **Paper III**). Further investigations of reasons for poor Se of faecal culture for detection of S. Dublin-infected cattle are warranted. Specificity (Sp) of faecal culture is usually assumed to be 100% even though risk of cross-contamination or errors in registration of test-results might lower the diagnostic Sp slightly.

Even though the Se of faecal culture for detection of infected animals is far from perfect, it has been suggested that repeated faecal culture can be used to detect active carriers (House et al., 1993). Alternatively, an initial serological screening followed by faecal culture of all seropositive animals to detect active carriers for culling might be a more cost-effective option (Veling, 2004). In the face of the poor Se and fairly high cost of bacteriological culture, simulation studies are needed to assess cost-effectiveness of such methods for control of *S*. Dublin under different circumstances at herd level.

PCR for agent detection

Today faster and more sensitive methods based on detection of genetic material have been developed for detection of *Salmonella* bacteria in food products, environmental samples and faecal samples (Kongmuang et al., 1994; Fratamico, 2003; Persson et al., 2012). There are two main principles in PCR-methods: the traditional PCR and real-time PCR. In the traditional PCR the test result is qualitative (yes/no). In real-time PCR the amount of copied DNA is counted by a computer after each cycle by the use of fluorescent probes. The first cycle where fluorescence becomes higher than the background is called the threshold cycle (Ct). The Ct-value is therefore inversely correlated to the starting concentration of target DNA in the sample. The performance of PCR-tests depends a lot on well-functioning primers and probes, and internal controls are important. The PCR-tests do not all provide information about the *Salmonella* serotype detected, so follow-up bacteriological culture has to be performed on positive samples to attempt to isolate the bacteria, if serotyping is desired. Most published studies report on test results from samples that were under suspicion for containing *Salmonella* or spiked samples, but lack of information about concentration of bacteria in the samples or appropriate negative reference groups make it difficult to assess the true Se and Sp of these methods for detection of infected animals. In a study on samples from naturally infected cattle with low concentrations of *S*. Dublin in faeces, rt-PCR was found to have poorer Se than the conventional faecal culture method (Jensen et al., 2013).

Molecular methods (e.g. plasmid analysis and pulsed-field gel electrophoresis), and genomic typing (e.g. ribotyping and IS*200*) for differentiation of *S*. Dublin strains are available (Olsen, 2005). These methods are, however, mainly used for academic purposes, and it is beyond the scope of this review to go into detail with these methods

Detection of antibodies

Enzyme-linked immunosorbent assays (ELISAs) measuring the level of immunoglobulins (antibodies) directed against O-antigens from *S*. Dublin in blood and milk can be used to measure the humoral immune response as an indicator of current or previous infection (Robertsson, 1984). The first time an animal becomes infected it takes one to a few weeks for the IgG levels to rise to measurable levels. Due to the feasibility of sampling and low costs of testing this test method is still useful for making probability diagnoses for surveillance, decision support and evaluation of control efforts. The ELISAs used for *S*. Dublin in Denmark report the analysis results as a so-called ODC%-value, which is a background corrected proportion of the test sample optic density (OD) to a positive reference sample. The ODC% should be interpreted as a semi-quantitative measure of the level of antibodies rather than a specific concentration of antibodies in the sample. In principle, ODC% can be negative if the level of antibodies in the sample is lower than in the negative control samples. In practice, the scale is often truncated to 0 and rarely goes above 150 ODC% (Nielsen and Ersbøll, 2004).

Bulk-tank milk ELISA

In Danish dairy herds the bulk-tank milk (BTM) is collected either every day or every other day. Through the mandatory milk quality control scheme BTM samples are routinely collected by dairy truck drivers from all dairy farms either every time milk is collected (more than 90% of the dairy farms) or weekly depending on which dairy company the herd delivers milk to. The BTM is tested for somatic cell counts, fat and protein content and farmers are paid on the basis of these parameters. Every third (from 2011 every fourth) month BTM samples from all dairy farms are tested in the national surveillance programmes for *S*. Dublin, BVDv and Infectious Bovine Rhinotracheitis (IBR). BTM is a convenient pooled sample from dairy herds and it facilitates very cheap surveillance options. However, it has some limitations because the milk from high-titre cows is diluted by the milk from low-titre cows. The dilution effect differs with herd size and within-herd prevalence of *Salmonella* (Nielsen and Ersbøll, 2005). Furthermore, BTM only includes measurements on lactating cows. Hence, it does not provide indications about infection in young stock (Veling et al., 2002). In herds with separated barn areas for young stock and cows the predictive value of the surveillance classifications based on BTM monitoring may therefore be low.

The following factors have been associated with increasing BTM *S*. Dublin ODC%: Positive *S*. Dublin or *S*. Typhimurium bacteriological status of the farm based on faecal sampling of all animals, mean yield-corrected antibody measurements in individual cows and number of high-titre cows (>80 ODC%). The level of antibodies in BTM is strongly associated with spread of infection among cows (Wedderkopp et al., 2001; Nielsen and Ersbøll, 2005). In the Netherlands, the Se of a single BTM ELISA measurement for detection of *S*. Dublin infected dairy herds was estimated to 38% at cut-off point OD=0.4, which is approximately equivalent to 25 ODC% in the test used in Denmark (Veling et al., 2002). In that study, 30 of 79 dairy herds known to be infected with *S*. Dublin were positive in BTM collected 2 to 4 months after the outbreak of *S*. Dublin was recognized. At lower cut-off the Se was higher, but even at the lowest cut-off OD=0.1, the Se of the BTM ELISA was only 68.4%. The Sp was estimated at cut-off point 0.4 to be 98.4% in 125 Dutch control herds that had no history of salmonellosis. In the Danish surveillance programme for *S*. Dublin in dairy herds the classification of herds is based on the average ODC% of four consecutive BTM samples and the ODC% in the most recent sample compared to the average of the previous three. The Se and Sp were estimated to be 95% and 96%, respectively, at an underlying true prevalence of 15% infected herds using this more complex combination of *S*. Dublin BTM antibody measurements over time (Warnick et al., 2006 **Paper XIV**).

Individual milk ELISA

Approximately 90% of all dairy herds in Denmark participate in a voluntary milk recording scheme in which milk samples are collected from all lactating cows either six or eleven times per year. Through this system the farmer can easily order testing of *S*. Dublin antibody measurements in the same samples either once or on a regular basis over a period of time. The accuracy for detection of infected animals of the individual milk (IM) ELISA, which is essentially analysed in the same way as BTM samples, has been evaluated by two methods i) a classical approach in which faecal culture results from the same animals are used as reference standards and ii) a latent class approach which estimates Se, Sp and prevalence through a mathematical optimization procedure in two population groups. The IM ELISA Se at cut-off 25 ODC% was estimated to 77% -78% and the Sp to 65%-86% depending on which test evaluation method was used. At cut-off 50 ODC%, these estimates were 42%-43% and 81%-94%, respectively, suggesting a marked drop in Se for detection

of infected animals at the high cut-off compared to the low cut-off (Nielsen, 2003). In practice it may be beneficial to interpret IM ELISA results from repeated measurements of cows rather than single ELISA test results when using this test method to control *S*. Dublin in dairy herds (Smith et al., 1989; Nielsen et al., 2004a).

Serum ELISA

The serum ELISA that is used in Denmark has been evaluated in two studies. The Se for detection of infected cattle is similar to the IM ELISA. At cut-off 50 ODC% the estimated Se is 45%-74% and Sp is 89%-100% depending on the age of the animal and the estimation method used in the study (Nielsen and Ersbøll, 2004; Nielsen et al., 2004b). Lowering the cut-off increases the Se and lowers the Sp. The test performs best in animals above 3 months of age and below approximately 10 months of age. In calves <3 months old serology is not recommended (Da Roden et al., 1992; Nielsen and Ersbøll, 2004). The Se estimates indicate the probability that the test will be positive given that the animal is truly infected, but not necessarily currently shedding bacteria. The Se of the individual ELISAs for detection of infected animals is thus markedly higher than that of the faecal culture methods. In practice, culling of cows and heifers with repeatedly high antibody results in serum or milk is only feasible, when prevalence is low in the herd (Nielsen and Dohoo, 2011 **Paper XVI**).

Conclusions and perspectives

The literature on elements of the pathogenesis of *Salmonella* infections is abundant. However, there are relatively few in vivo studies on *S*. Dublin pathogenesis in cattle which include more than a few animals, and the studies often show varying or conflicting results. It makes it difficult to draw conclusions about mechanisms that affect dissemination in cattle and that might be targets for control methods directed towards improving resistance against the bacteria, e.g. new vaccines. It is therefore recommended to perform larger studies to elucidate dose-response relationships and age- and genetic effects of immunity. Synergy in new knowledge creation and knowledge transfer can potentially be improved by joining the host and agent-level pathogenesis studies with observational studies investigating the effect of herd level immunity on the spread and clinical expression of *S*. Dublin infection under natural farm conditions.

It is recommended to develop faster and more sensitive methods for detection of *S*. Dublin for diagnosis of infectious animals. This would also facilitate the observational studies suggested above. In the face of the poor Se and fairly high cost of bacteriological culture, simulation studies are needed to assess cost-effectiveness of existing and new diagnostic methods and procedures for control of *S*. Dublin under different circumstances at herd level.

Acknowledgements

Professor John Elmerdahl Olsen and Professor Søren Saxmose Nielsen from Faculty of Health and Medical Sciences, University of Copenhagen, Denmark, are gratefully thanked for critical comments to this review.

References

References used in this paper are listed after Chapter 8 together with references used in Chapters 3 to 8.

Chapter 3
Occurrence

This chapter summarises the results from observational studies reported in papers II to V investigating *S.* Dublin herd level and within-herd prevalence, incidence and seasonality in dairy herds, and herd level and within-herd prevalence of *S*. Dublin in non-dairy herds. Information on the accuracy and predictive values from Chapter 6 on test-strategies and surveillance are used in the calculations and estimations in this chapter, but will be described in more detail in Chapter 6.

Background

An essential starting point in the surveillance and control of an infection is to know of the occurrence of the infection. If surveillance of a herd level occurrence in a country or region is based only on the submission of samples from clinical suspicions (i.e. passive surveillance), the true prevalence of an *S*. Dublin infection and disease caused by the infection is very likely to be underestimated. Hence, a representative survey of the target population should be performed for a prevalence estimation and should be repeated once or several times to be able to investigate the spatiotemporal patterns of the infection.

Before the initiation of the Danish surveillance programme for *S*. Dublin in 2002, knowledge of *Salmonella* occurrence at herd level came from passive surveillance based on the submission of faecal samples, aborted foetuses and other types of sample materials submitted by local veterinarians for bacteriological culture upon any suspicion of salmonellosis as required by the Danish salmonella legislation (Anonymous, 1996).

Figure 3.1 shows the number of cattle herds with clinical disease and *S*. Dublin positive bacteriological cultures and the human *S*. Dublin cases in Denmark between 1979 and 2011. In principle, the passive surveillance data might provide an indication of the incidence of *S*. Dublin. However, it would require that:

1) *S*. Dublin always produces clinical signs in infected animals that are in newly infected herds;
2) farmers always notice such clinical signs;
3) farmers always call the veterinarian for assistance upon seeing the clinical signs;
4) veterinarians always suspect salmonellosis when seeing clinical signs produced by *S*. Dublin;
5) veterinarians always submit samples upon suspicion;
6) the laboratory is able to culture *S*. Dublin bacteria from the submitted samples, record the results and make these results available for analysis.

Too many factors influence these preconditions to obtain reliable prevalence and incidence estimates from the passive surveillance activities for *S*. Dublin, e.g. the pathogenesis and dynamics of the infection with intermittent and low concentrations of the bacteria shed by infected cattle, and the imperfect diagnostic test sensitivity of the bacteriological culture (Nielsen, 2013 **Paper I**). Furthermore, changes in compensation practices in the cattle industry, consequences of *S*. Dublin isolations in relation to outbreaks, and structural changes in the Danish cattle population with a rapid change towards fewer and larger cattle herds make it difficult to interpret the numbers in Figure 3.1 (Wedderkopp, 1996).

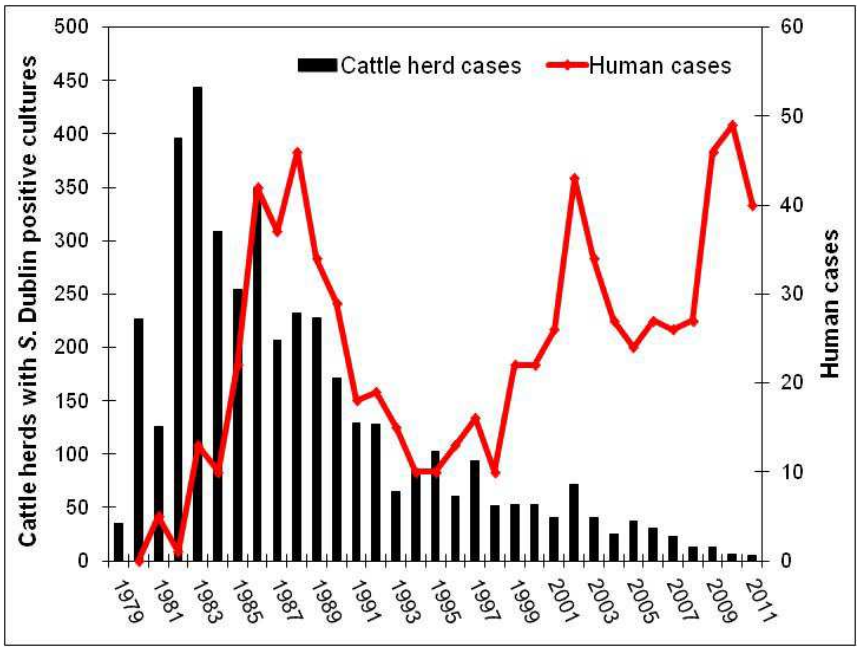

Figure 3.1 The number of Danish cattle herds diagnosed with salmonellosis diagnosed as *S*. Dublin and the number of human *S*. Dublin cases recorded in Denmark between 1979 and 2011 (Source: National Food Institute, Technical University of Denmark and the Knowledge Centre for Agriculture, Cattle).

A survey was performed from late 1994 to early 1996 in approximately 10% (n=1,464) of the dairy herds in 19 regions of Denmark to investigate the prevalence in dairy herds based on antibody BTM sampling in three testing rounds and the correlation with diagnoses made based on clinical suspicion during the same years (Wedderkopp et al., 2001). Around 74% of the tested herds were test negative in all three of the testing rounds, 10% tested positive in all of the rounds, 4% changed from test positive to test negative, 8% changed from test negative to positive and 4% had fluctuating BTM antibody results. There were indications that the prevalence was increasing over the test period. Furthermore, the percentage of dairy herds that changed from test negative to positive was significantly correlated to the estimated proportion of clinical cases within each region. On average there were four times as many herds becoming test positive as there were herds with clinical suspicions, and nine times as many dairy herds that were either test positive in all of the test rounds or changed to a test positive status (Wedderkopp et al., 2001). In conclusion, the knowledge of the true herd level incidence and prevalence of *S*. Dublin was inadequate.

Herd level prevalence in dairy herds

In the Danish national surveillance programme, dairy herds are classified into one of two levels, Level 1 (test negative) and Level 2 (test positive or recently in contact with test positive herds), based on antibody measurements in the most recent and the previous three year-quarterly collected BTM samples. However, the proportion of test positive dairy herds in the programme overestimates the true prevalence of infected dairy herds. This was shown in a study that estimated HSe, HSp and predictive values at different underlying assumed true prevalence of infection (Warnick et al., 2006 **Paper XIV**). The study will be described in more detail in Chapter 5 with information on the test-strategies and the surveillance. To give an example from that study,

the estimated HSe (i.e. the probability that a dairy herd would be classified as Level 2 in the surveillance programme due to high antibody levels given that the herd was infected) was 95% regardless of the level of the underlying true prevalence. At a true prevalence of 15% *S*. Dublin infected dairy herds, the positive predictive value (PPV) was on average 80% indicating that 20% of the test positive dairy herds in regions with 15% infected herds were misclassified, in many cases because they had been infected and had cleared the infection, but still had antibodies in BTM. The HSp was on average 96%, and the negative predictive value (NPV) was estimated to be 99% suggesting that only 1% of Level 1 herds were truly infected. In the paper by Nielsen et al. (2007 **Paper VI**) it was shown that the national apparent prevalence (AP) according to the surveillance programme testing procedures was 26% test positive dairy herds at the end of 2001. By mid 2003 it had dropped to 22%, but there were large regional differences as illustrated in Figure 3.2.

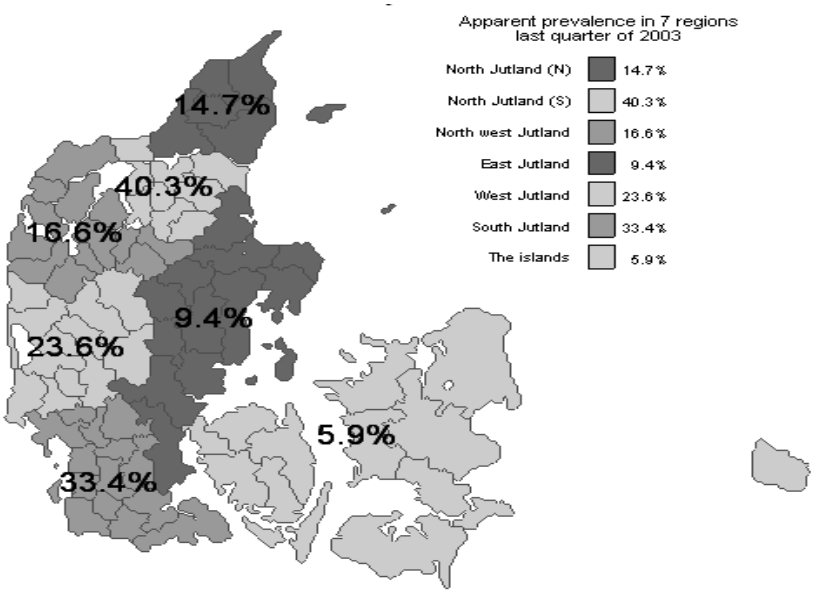

Figure 3.2 Apparent prevalence distribution in dairy herds in 7 regions of Denmark in June 2003 according to the herd classifications in the national surveillance programme for *S.* Dublin in cattle (Nielsen et al., 2007 **Paper VI**).

To illustrate the change in herd level true prevalence (TP) over time, while correcting for the misclassification in the national surveillance programme, I have used the HSe and HSp estimates from Warnick et al. (2006 **Paper XIV**) to calculate the true herd level TP in dairy herds for every quarter of the year since 2002 using the AP from the surveillance programme antibody testing scheme (i.e. not including the official herd classifications based on trade). The TP was calculated using the simple adjustment method in Eq. 3.1 (Houe et al., 2004):

$$TP = (AP+HSp-1) / (HSp+HSe-1) \qquad \text{Eq. 3.1}$$

In Eq. 3.1 HSe was set to 0.95 regardless of TP and AP, HSp was set to different values depending on the underlying TP that would be obtained with the given numbers as shown in Table 3.1. Figure 3.3 illustrates the change in the number of active dairy herds (N) and the herd level TP and AP over time. Figure 3.4 illustrates the development in TP between seven regions in Denmark.

Table 3.1 Herd specificity (HSp) parameters used to calculate the true prevalence (TP) of *S*. Dublin in Danish dairy herds from the given apparent prevalence (AP) values based on bulk-tank milk (modified from Warnick et al. (2006 **Paper XIV**)).

AP	HSp	TP	AP	HSp	TP
0	1	0.00	0.28	0.94	0.25
0.01	0.99	0.00	0.30	0.93	0.26
0.02	0.98	0.00	0.32	0.93	0.28
0.04	0.98	0.02	0.34	0.92	0.30
0.06	0.98	0.04	0.36	0.92	0.32
0.08	0.98	0.06	0.38	0.91	0.34
0.10	0.97	0.08	0.40	0.91	0.36
0.12	0.97	0.10	0.42	0.90	0.38
0.14	0.97	0.12	0.44	0.89	0.39
0.16	0.96	0.13	0.46	0.88	0.41
0.18	0.96	0.15	0.48	0.87	0.43
0.20	0.96	0.18	0.50	0.86	0.44
0.22	0.95	0.19	0.52	0.85	0.46
0.24	0.95	0.21	0.54	0.84	0.48
0.26	0.94	0.22	0.56	0.83	0.50

The difference between AP and TP changed over time from at most 3.1%-points to 1.4%-points. Due to the distinct fall in the number of dairy herds, this meant that the estimated number of antibody positive dairy herds that were not truly infected went from 261 (14%) out of 1,895 in 2002 to 57 (17%) out of 343 antibody positive herds in early 2012. In other words, the PPV was reduced, but the absolute number of herds that were falsely positive for *S*. Dublin was markedly reduced as the true prevalence and the number of active dairy herds went down. It is important to make these extra calculations rather than just consider the apparent prevalence when making decisions about new or adjusted control measures at a national level.

It is difficult to find reliable herd level prevalence estimates from other countries to compare to, either because no systematic and representative collection of data has been done (Anonymous, 2009b), or because the surveillance is based on bacteriological sampling of fewer samples leading to a poor HSe.

Sweden has a system based on a mandatory notification of all of the *Salmonella* detections and the strict handling of all of the infected herds regardless of serotypes combined with a back-tracing of the infections. The prevalence is apparently so low, and the handling of the infected herds is so intensive and probably effective in removing *Salmonella* from the premises, that it makes more sense to talk about incidence rather than the prevalence of *Salmonella* in cattle herds in Sweden. On the other hand, the apparent incidence ranged from 5 to 23 new *Salmonella* infected cattle herds per year between 1993 and 2010, 62% of those being *S*. Dublin (Lewerin et al., 2011), which is similar to the numbers seen in Denmark today where the TP is around 7% according to Figure 3.3. There appears to be a geographical area in the South East of Sweden with an endemic infection of *S*. Dublin (Lewerin et al., 2011). In such regions, active surveillance would be useful to detect subclinically infected herds in order to try to reduce the persistence of infection in the herds and prevent further spread to other herds.

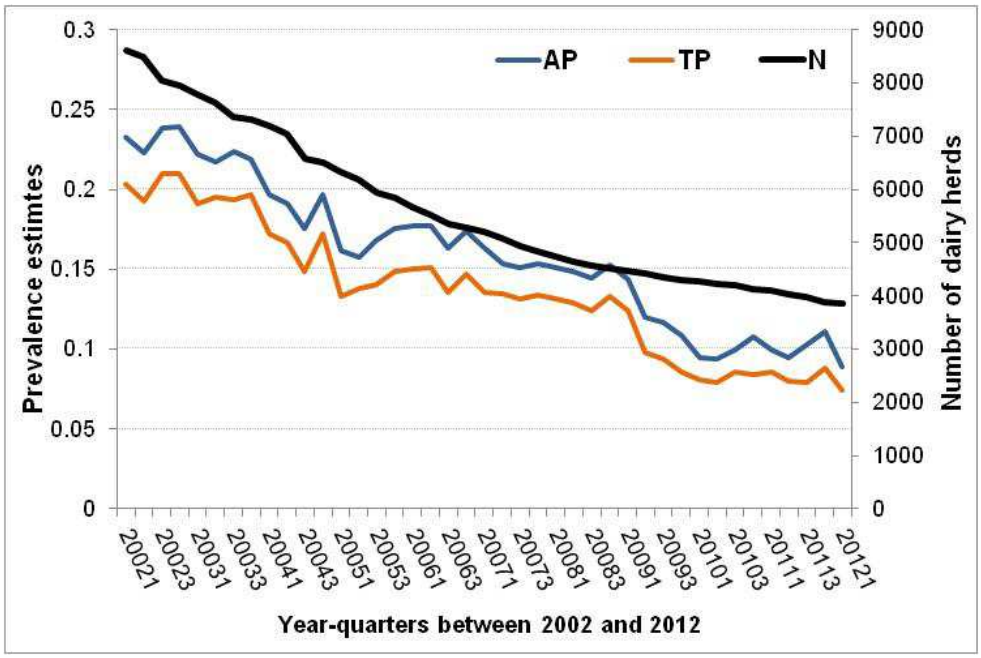

Figure 3.3 The number of active dairy herds (N), apparent (AP) and true prevalence (TP) estimates of *S*. Dublin in Denmark from 2002 to 2012.

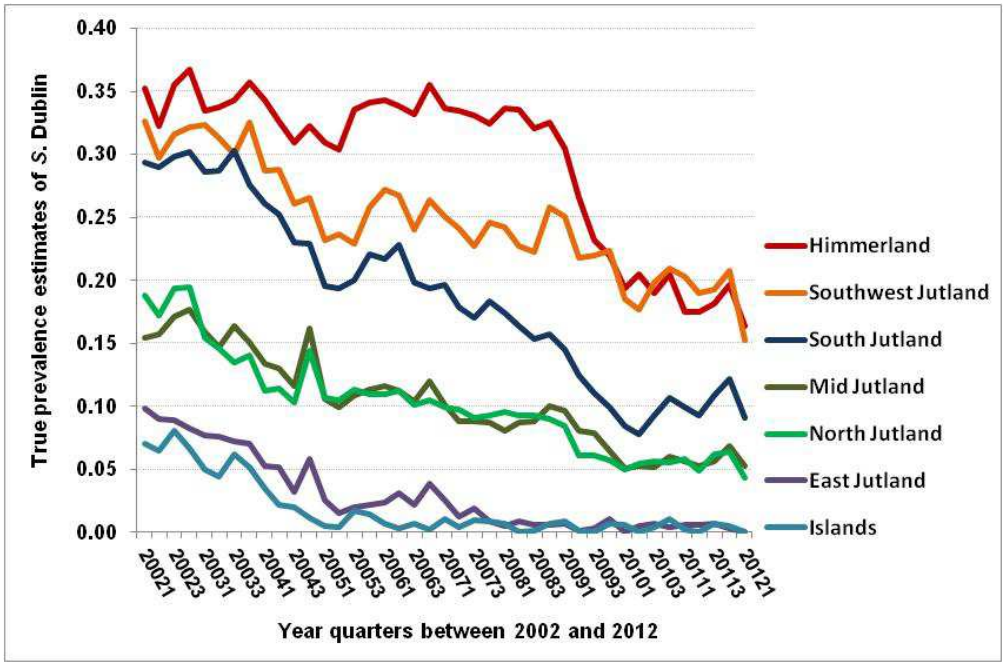

Figure 3.4 The development in true prevalence estimates of *S*. Dublin in dairy herds in seven regions of Denmark from 2002 to 2012.

In the Netherlands, the estimated national true prevalence of *Salmonella* in dairy herds was reported to be 8.3% (95%CI: 0.7-15.8) in 2004, based on a random sample of 143 dairy herds (Bergevoet et al., 2009). The prevalence was based on ELISA-testing of a single BTM sample from each herd. The ELISA detected *Salmonella* serogroups B and D antigens, and the cut-off was such that a within-herd prevalence of >10% would be detected (Dr. Gerdien van Schaik, Animal Health Service, Deventer, The Netherlands, personal communication, 8th August 2012). The antigen used in the Danish surveillance programme is a serogroup-D antigen, which is supposed to make it more specific for *S*. Dublin than the test used in the Netherlands. However, cross-reactions with other *Salmonella* serotypes can occur (Konrad et al., 1994; Nielsen, 2003).

A survey based on conventional bacteriological culture of pooled faecal pat samples and slurry samples from a relatively large number of randomly selected dairy herds (n=443 at first round of sampling) in England and Wales in 1999-2001 demonstrated that one or more *Salmonella* serovars was detected in 12% to 24% of the herds. *S*. Dublin was detected in 3.3% to 6.7% of the farms depending on the season (Davison et al., 2005).

In Western France, a survey of 489 dairy herds in 2001 to 2003 also based on conventional bacteriological culture of stored manure and slurry found 8% (95%CI: 5-13%) of the herds positive for *Salmonella* spp. However, *S*. Dublin was not isolated from any of the herds in that study (Lailler et al., 2005). Knowing the low number of samples collected per herd and the poor sensitivity of the test method used for this study, it is not unlikely that endemic *S*. Dublin infections in some of these herds were overlooked (Veling et al., 2002; Jensen et al., 2013).

In conclusion, very few studies which provide reliable herd level prevalence estimates of *S*. Dublin in dairy herds were identified, and most of those that are available underestimate the true prevalence. Studies included in this thesis (Warnick et al., 2006 **Paper XIV**; Nielsen et al., 2007 **Paper VI**) have contributed considerably to provide a detailed knowledge about herd level prevalence in Danish dairy herds, including any regional and national changes over time as summarised in Figures 3.3 and 3.4.

Herd level incidence in dairy herds

Whereas the prevalence may be informative about the occurrence of an infection, the incidence provides an insight into the absolute number of herds that becomes infected within a given time period. The incidence rate describes the speed of the spread of infection, which is an important parameter in the control of infectious diseases. As argued above this parameter is difficult to obtain from passive surveillance data. However, the Danish systematic and longitudinal surveillance data provided a unique opportunity to investigate the incidence of *S*. Dublin infection in dairy herds (Nielsen and Dohoo, 2012 **Paper VII**). To the authors' knowledge this was the first paper to investigate the herd level incidence of *S*. Dublin in cattle herds based on observational data. Herds were considered at risk of new infection in the year-quarter (YQ) following four test negative YQs. Incidence risk was calculated as the number of new test positive herds divided by the number of herds at risk per YQ. Figures 3.5 and 3.6 are modifications of Figure 1 in Nielsen and Dohoo (2012 **Paper VII**) including two more years of data. It should be noted that the incidence of *S*. Dublin was reported for a period in which there was active surveillance in place from 2002 and an intensified control campaign in addition to surveillance from 2007 and onwards. This must be assumed to influence the incidence risk over time compared to a situation without surveillance

and control programmes. However, it is difficult to know what would have happened if these programmes had not been in place in Denmark.

Results of a study of clinical isolates of *Salmonella* in cattle herds under passive surveillance in Great Britain between 2003 and 2008 showed that the incidence risk remained fairly stable over time (slowly reducing the number of isolations of *Salmonella* together with a reduced number of cattle herds). The proportion of isolates that were *S*. Dublin reduced from 81% in 2003 to 64% in 2007. *S*. Typhimurium increased from 9% to 18%, and other serotypes increased from 10% to 19% between 2003 and 2007 (Carrique-Mas et al., 2010). Therefore, it is most likely that the incidence risk would have remained stable or fluctuated over time in a similar manner rather than decreasing as observed in Denmark (Figures 3.5 and 3.6) if the surveillance and control programmes had not been initiated. According to Carrique-Mas et al. (2010) the distribution of serovars responsible for salmonellosis has shifted somewhat over time. This is impossible to evaluate with the low number of submissions of samples from clinical suspicions in Denmark (Figure 3.1). Therefore, if we want to know the distribution of *Salmonella* serotypes in the Danish cattle population today and onwards, we need to perform field studies based on random sampling principles.

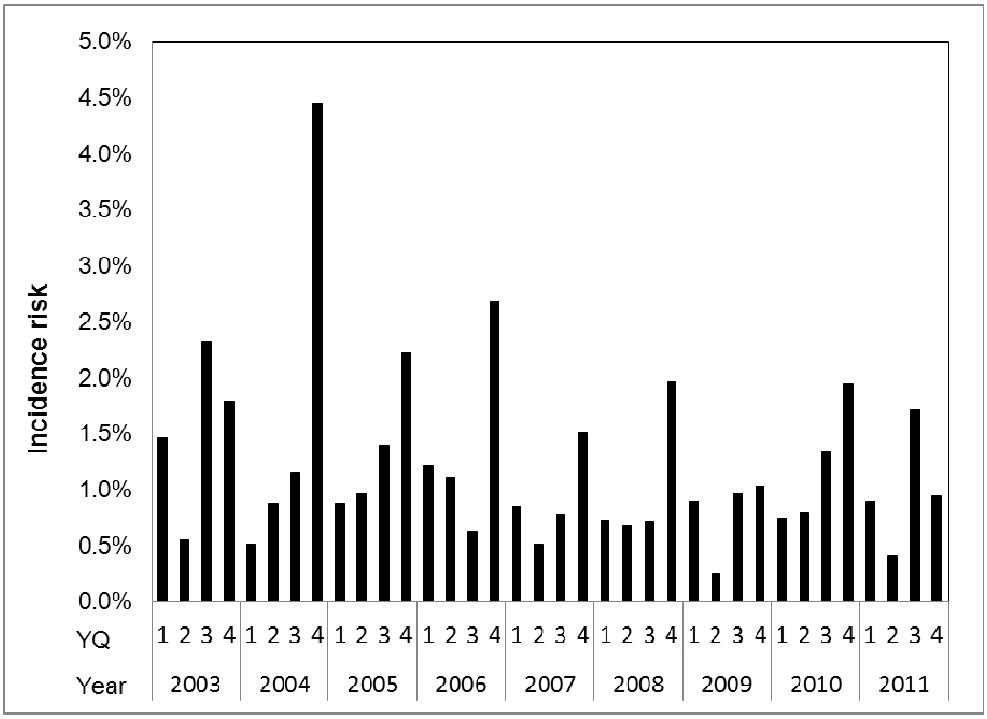

Figure 3.5 The incidence risk of *S*. Dublin based on data from the Danish surveillance programme from 2003 to 2011.

Seasonality

July to October is the period where the incidence of *Salmonella* infections in cattle is generally highest. This is also when *S.* Dublin outbreaks peak almost every year in the dairy cattle population (Nielsen and Dohoo, 2012 **Paper VII**). There have been fewer recorded outbreaks over the years,

but seasonality is obvious every year. The subsequent increase in antibodies in BTM may be delayed by at least 3 months, and therefore the incidence risks are highest in the fourth YQ of each year in Figures 3.5 and 3.6. A recent study from Great Britain found a similar pattern, and did not find any specific explanations for the seasonality in analyses of the effect of rainfall on the *Salmonella* incidence (Carrique-Mas et al., 2010). A study of Danish outbreaks of *Salmonella* in cattle herds from 1990 to 1998 indicated that there is a positive association between higher temperatures from May to August and higher numbers of isolations from clinical salmonellosis in cattle in Denmark (Steffensen and Blom, 1999).

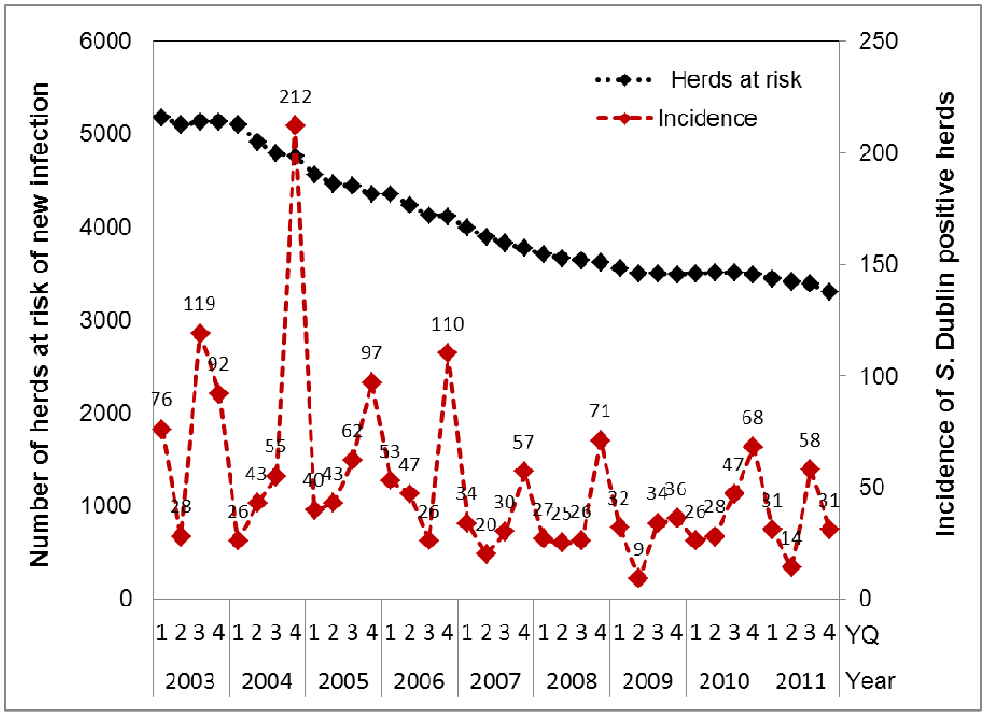

Figure 3.6 The number of dairy herds at risk of becoming infected, and the incidence of *S*. Dublin based on data from the Danish surveillance programme from 2003 to 2011 (modified from Nielsen and Dohoo (2012 **Paper VII**)).

Within-herd prevalence in dairy herds

In addition to the prevalence and the incidence at herd level, it is essential to understand the epidemiology of *S*. Dublin within the infected herds in order to plan diagnostic test strategies, surveillance and control programmes. However, there are very few studies that provide a good insight into within-herd prevalence and the dynamics of *S*. Dublin, in particular for persistently infected cattle herds. Veling et al. (2002) measured the seroprevalence in all age groups of cattle in 79 dairy herds 2 to 4 months after confirmed outbreaks of *S*. Dublin and found that the seroprevalence varied between 10% and almost 60%. Levels of seroprevalence above 30% were only seen in calves between 3 and 7 months old. The authors of that study also reported that the seroprevalence in young stock did not vary between herds with and without clinical signs, indicating that the serology of young stock is a good indicator of a subclinical *S*. Dublin infection. In adult cattle the seroprevalence was 12% on average in that study. However, a subsequent study of

some of the same herds showed large variations from 0 to 70% in both young stock and adults between herds (Veling, 2004).

One of the reasons for the varying levels of prevalence observed in the field studies is that *S*. Dublin is a very dynamic infection in cattle herds, and in some barn sections the cattle population is also very dynamic. This became evident in Nielsen et al. (2007 **Paper III**) in which the transmission parameters and temporal infection dynamics of *S*. Dublin were investigated in calves below 6 months old in four Danish dairy herds. Based on frequently (i.e. bi-weekly or weekly) repeated sampling, 181 calves were assigned to three infection compartments: susceptible (S), infectious (I) and recovered (R) based on faecal culture results and serum ELISA measurements. An environmental component was added to mimic the infections that occurred even when there were no calves classified as infectious present in the herd. As illustrated for one of the herds in Figure 3.7, the number of calves in the different compartments changes every week and the calves were moved in and out of the barn area as part of the ordinary management of calves of different ages. Small groups of calves may eventually "run out" of susceptible calves unless new calves are introduced into the group continuously, whereas large herds more frequently would have enough new susceptible calves coming into the herd to continuously to keep an *S*. Dublin infection persistent in the calf barn section.

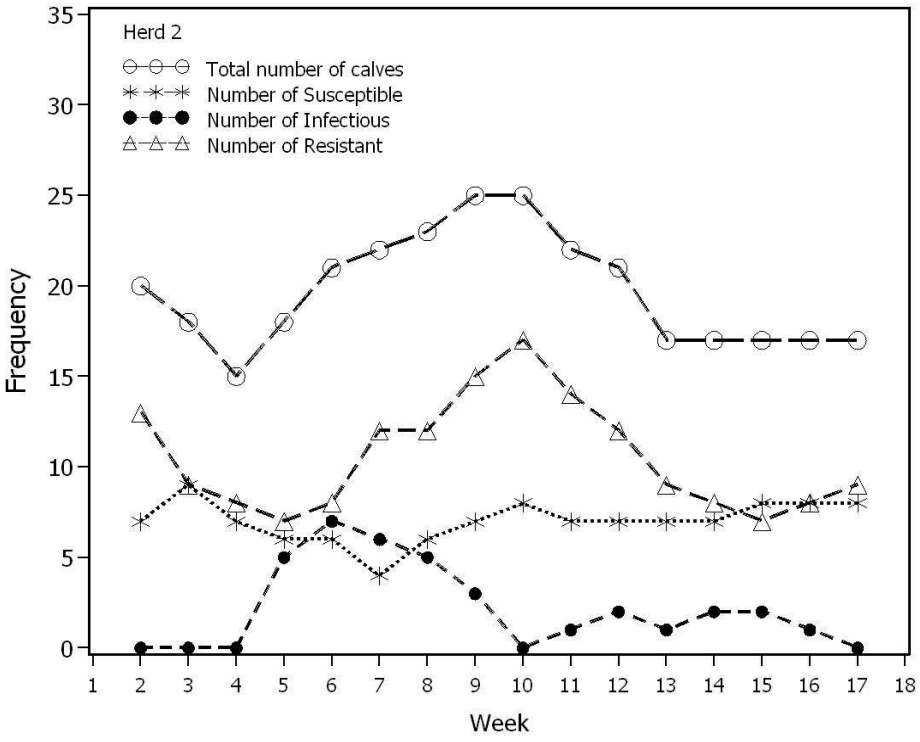

Figure 3.7 An illustration of the dynamic size of the compartments S, I, R and the total number of calves in per week in Herd 2 during the study period. There was a small outbreak of an *S*. Dublin infection in weeks 5 to 8. The large fluctuation in N is mainly due to bull calves being sold from the herd at around two weeks of age and the movement of calves between barn areas (Nielsen et al., 2007 **Paper III**).

In a study of a large Californian dairy herd with clinical problems the prevalence of seropositive adult cows was 3.5%, whereas in calves it was 52%. In that study, 11% of the calves were found to be faecal culture positive for *Salmonella* bacteria (House et al., 1993). Neither the herd size nor the management reported in that study were representative of the Danish dairy herds. Therefore analyses were performed on the data collected from 14 *S*. Dublin infected dairy herds from 2000 to 2002 (Nielsen, 2013 **Paper II**), which is the only study to date based on a large longitudinal and extensive data collection for an investigation of within-herd *S*. Dublin epidemiology. All of the herds were visited 5 times at approximately 3 month intervals (except one that was visited 4 times). All of the cattle present in the barns were sampled at each visit (i.e. animals on pastures were excluded from the sampling rounds) leading to a total of 10,162 paired samples for analysis of antibodies and bacteriology. In addition, serum samples were collected from all of the non-lactating cattle (calves, young stock and dry cows), and milk samples were collected from all of the lactating cows during the morning milking for analysis of antibodies directed against *S*. Dublin LPS as described in Chapter 2. A cut-off of 50 ODC% was used to differentiate between serologically negative and positive samples in the subsequent descriptive and statistical analyses. The sensitivity and specificity of these methods depended on age, as described in two previous papers (Nielsen and Ersbøll, 2004; Nielsen et al., 2004b).

Figure 3.8 shows the distribution of faecal positive samples collected from calves below 180 days old, young stock from 6-24 months old and adult cows. In general, there were very few faecal positive samples in the herds. Only during 4 out of the 69 visits did the prevalence of faecal culture positive cattle exceed 10%. The number of positive faecal samples at each visit varied between 0 and 6 in the calf group, 0 and 7 in the young stock, and 0 and 5 among the cows. For a comparison the number of collected samples at each visit varied from 9 to 61 (mean=26) in the calf group, 2 to 103 (mean=39) in the young stock, and 18 to 154 (mean=83) in the cows. One herd had faecal positive samples from the adult cows during 4 visits, but no positive samples from the calves or the young stock. Seven herds only had positive samples from the calves and the young stock, but not from the adult cows. Two herds only had 1 positive sample during one visit, one from the calves and the other one from the young stock. Three herds had positive samples from all of the age groups, but only one herd had *S*. Dublin isolated from all of the barn sections on the same day. All in all these results showed that the occurrence of *S*. Dublin in these endemically infected herds was difficult to describe accurately using only the faecal culture of faecal samples. This is related to a poor diagnostic sensitivity, which is due to the intermittent and low-concentration excretion characteristic of *S*. Dublin in cattle after the initial acute phase of the disease (Robertsson, 1984; Steinbach et al., 1996; Jensen et al., 2013).

Figure 3.9 shows the distribution of levels of seroprevalence in rolling age intervals among cattle under 2 years old and adult cows across all of the 69 visits to the 14 dairy herds. In the young stock, the pattern was similar to that reported by Veling et al. (2002) up until 7 months. The seroprevalence decreased from on average 17-18% in new born calves to 5% in 2 months old calves. This pattern reflects the decline in maternally derived antibodies combined with the fact that calves below 11-12 weeks old have a poor ability to produce antibodies against the infection (Da Roden et al., 1992; Nielsen, 2003). The seroprevalence then rapidly increased until it peaked at around 5-6 months of age. After 4 months of age the seroprevalence was generally between 25-35% in most of the heifers and adult cows, except from 11 to 15 months where the seroprevalence appeared to be reducing towards 15% (Nielsen, 2013 **Paper II**).

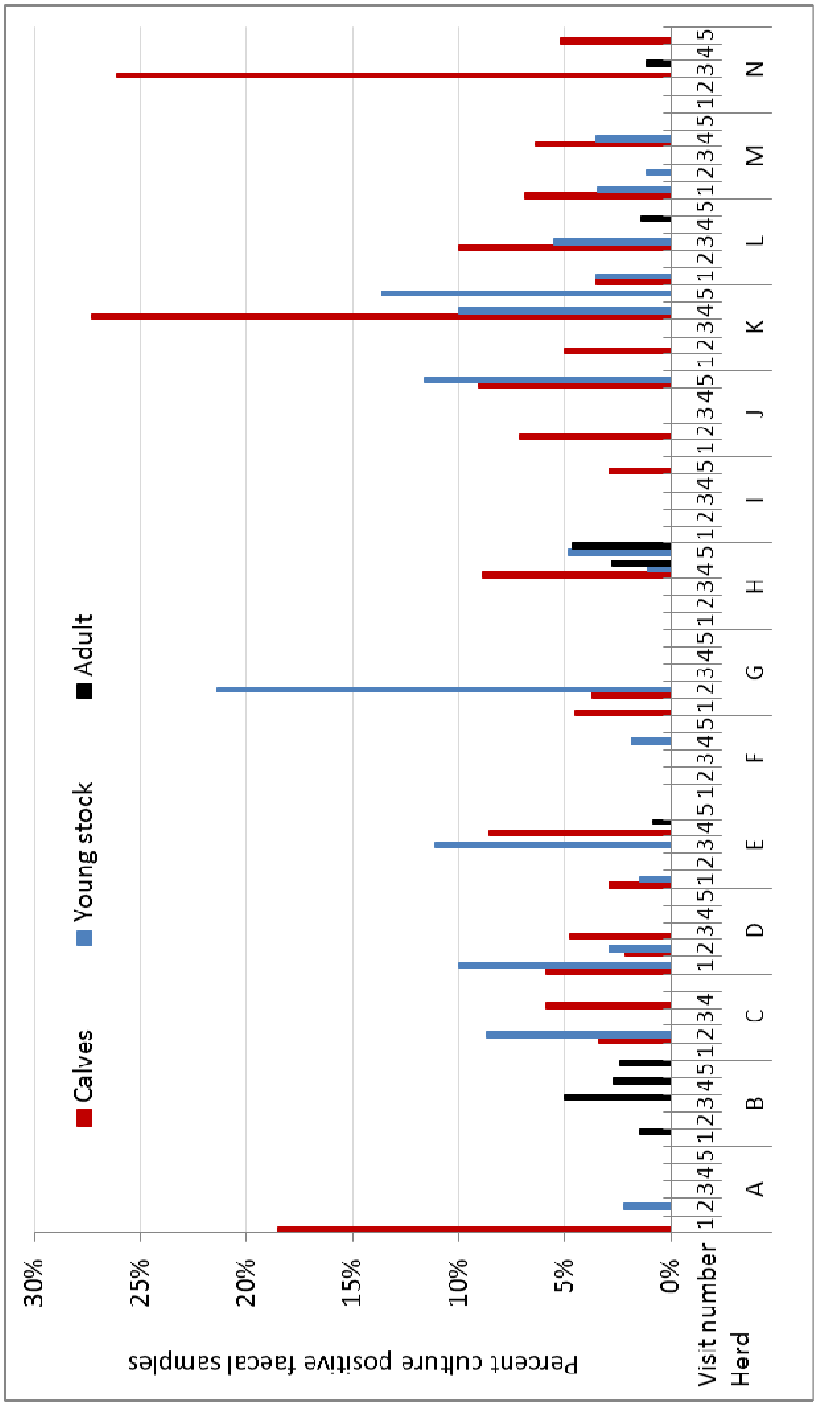

Figure 3.8 The prevalence of *S.* Dublin-faecal positive samples in calves, young stock and adult cattle in 14 endemically infected dairy herds (A-N) that were visited 4 to 5 times each within 3 month intervals during 2000 to 2002. The visits are shown along the x-axis as 1-5.

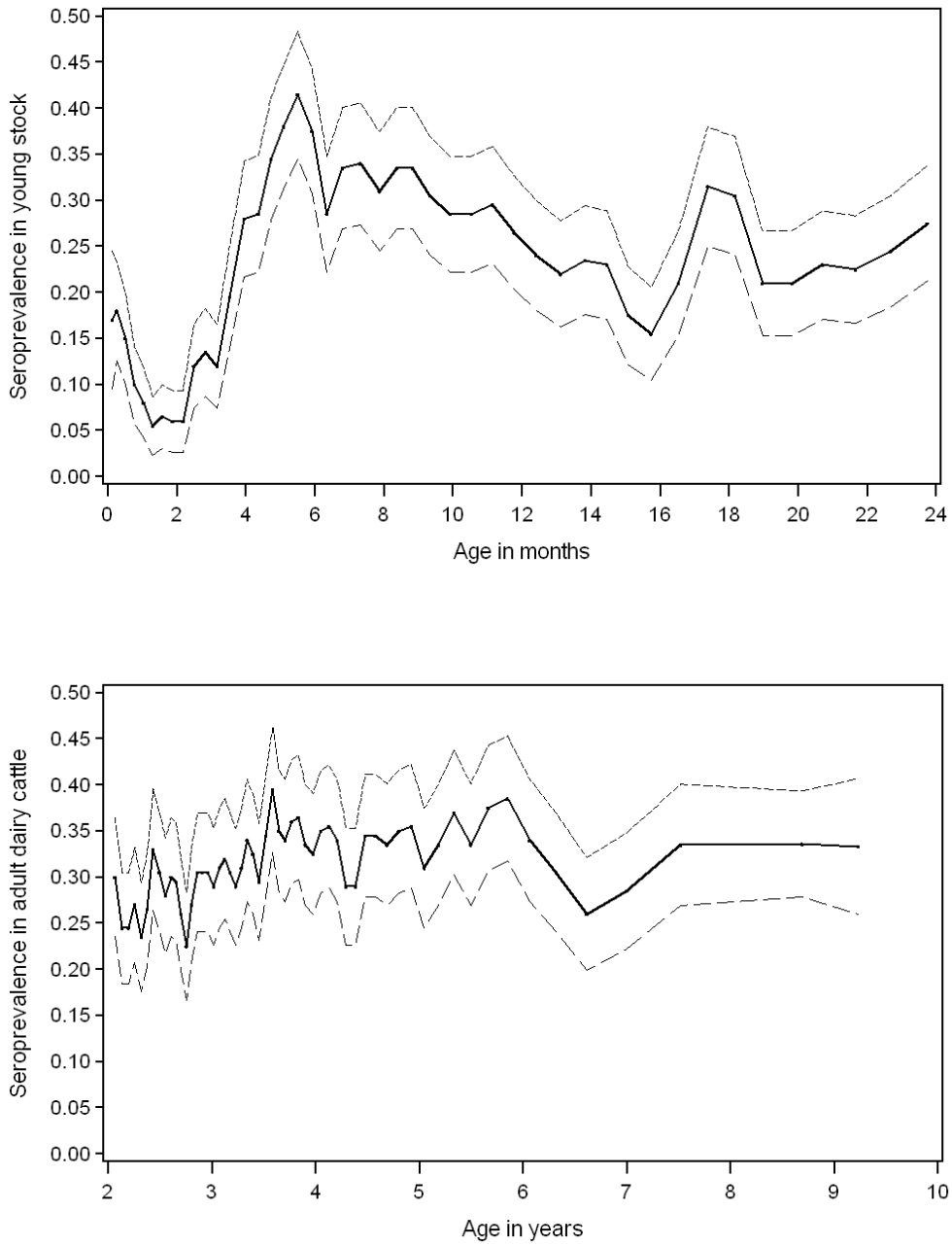

Figure 3.9 The seroprevalence in young stock (top) and adult cows (bottom) in 14 endemically *S*. Dublin infected dairy herds tested repeatedly during 2000-2002. The solid lines show the mean seroprevalence and the dashed lines show the 95% confidence intervals. The points represent the mean in rolling age intervals that each contains 200 observations (modified from Nielsen (2013 **Paper II**).

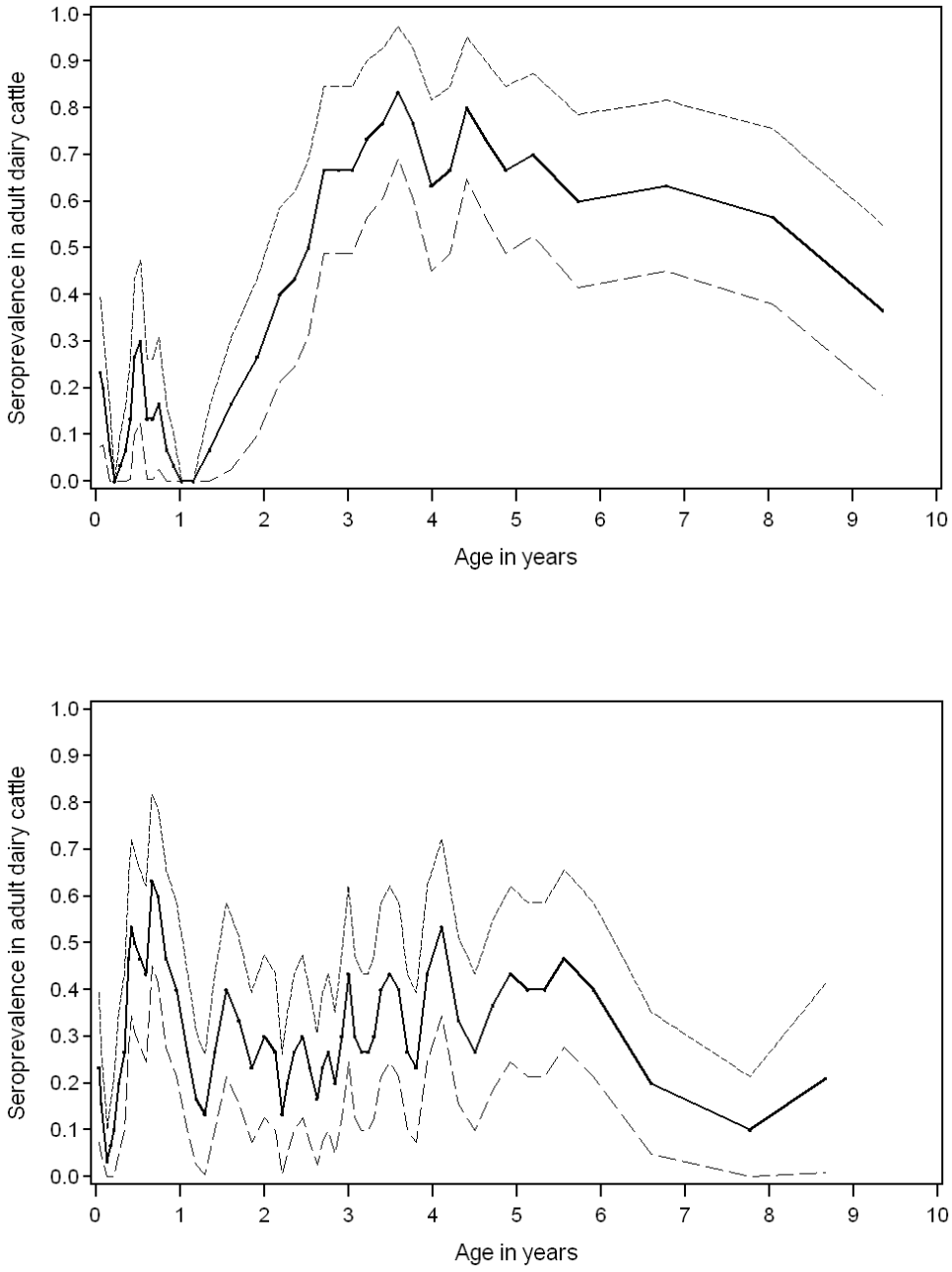

Figure 3.10 The seroprevalence measured in all of the age groups of cattle in 2 endemically *S*. Dublin infected dairy herds. The solid lines show the mean seroprevalence and the dashed lines show the 95% confidence intervals. The points represent the mean in rolling age intervals that each contains 200 observations. Both of the herds had *S*. Dublin positive faecal cultures in all of the age groups during the study period.

The seroprevalence in adult cows, hovering at around 25-35%, was generally a lot higher in adult cows than the 12% and 3.5% reported by Veling et al. (2002) and House et al. (1993), respectively. Further descriptive and statistical analyses at herd, age group and seasonal level revealed large variations between herds as illustrated for two herds in Figure 3.10. For variation and to provide more information than it is acceptable to include in a journal paper, Figure 3.10 illustrates two other herds than those displayed in Nielsen (2013 **Paper II**). The seroprevalence was significantly associated with the occurrence of positive faecal cultures in the calves and the adult cows, but not in the young stock. This suggests that calves (3-6 months old) and adult cattle are the best indicator groups of an actively spreading infection in herd classification procedures.

In all likelihood, an explanation of the observed differences between the herds could be found in the housing facilities, hygiene levels and management routines (Nielsen and Nielsen, 2012 **Paper XV**; Nielsen et al., 2012 **Paper IX**; Nielsen and Dohoo, 2013 **Paper VIII**; Nielsen et al., 2012b).

Herd level prevalence in non-dairy herds

Cattle herds that do not deliver milk (non-dairy herds) in Denmark are more heterogeneous with regard to their herd size, breeds, age and gender distributions, housing, grazing practices and management than dairy herds. Thus, a good method for herd classification is much more difficult to decide than for dairy herds, and herd level prevalence estimates for non-dairy herds mainly come from surveys targeted towards specific subgroups of non-dairy herds, often feedlots or specialised veal-calves.

Nielsen et al. (2011 **Paper IV**) estimated the prevalence based on the faecal culture sampling of calves sent to slaughter. Samples were collected at the slaughter line just after the intestines had been removed from the carcass. The herds mainly consisted of slaughter calves born in dairy herds and sold young to be raised in specialised veal producing herds (sometimes referred to as 'dairy beef herds'). The study used a random sample of herds that expected to send more than 100 calves to slaughter per year. The apparent *Salmonella* herd level prevalence was 18% (95%CI: 9-27) with 12 of the 13 test positive herds out of 71 study herds being *S*. Dublin positive and only one being *S*. Typhimurium positive. It was, however, expected that this was an underestimation of the true prevalence of *Salmonella* infected herds due to the poor sensitivity of faecal culture (Nielsen et al., 2004b). Therefore, another study was performed in which a Bayesian analysis was used to evaluate the within-herd and between-herd *Salmonella* prevalence in 68 of the herds from Nielsen et al. (2011 **Paper IV**) that had sufficient data to be analysed. The advantage of a Bayesian analysis is an avoidance of bias in the prevalence calculations by not using fixed (and often unknown) test accuracy estimates. Furthermore, estimates of the true prevalence and the test accuracy can be obtained simultaneously. Serological test results from 753 animals and faecal culture results from 1233 animals were available from these 68 herds. The median faecal culture Se was estimated to be 9% with a 95% credibility interval of 5-17% in the analyses which is very similar to the estimates obtained previously in subclinically infected cattle (Nielsen et al., 2004b). The ELISA Se was estimated to be 69% and the Sp to be 90%. These estimates also correspond well to those found in previous studies (Nielsen and Ersbøll, 2004; Nielsen et al., 2004b). The median Sp of faecal culture was estimated to be 100% (95% credibility interval: 99-100%). The estimated true herd level prevalence was between 34% and 57%, which was markedly higher than the 18% estimated in the study relying only on bacteriological cultures (Nielsen et al., 2011 **Paper V**).

Together, these two studies are the only studies providing reliable estimates of the herd level prevalence of *S*. Dublin in specialised veal producers in Denmark to date. The studies highlight the potential bias implicit in studies based solely on bacteriological culture methods. Different interpretations are possible, e.g. one might argue that the faecal culture test detects active shedders of bacteria, so that the study relying only on faecal culture provides an estimate of the prevalence of herds that pose the most important risk to food safety at slaughter. The TP estimated by a Bayesian analysis of both serological and culture tests might estimate a latent herd-infection status with a few actively shedding animals, but may also include many calves that have been exposed recently and therefore have antibodies directed against the infection at sampling. Due to the tendency of *S*. Dublin to persist in cattle herds, it is reasonable to consider herds that are serologically positive as infected and at risk of spreading the infection to other herds, the environment and through the food chain, not least in relation to control or eradication programmes.

There are still no true herd level estimates from other types of non-dairy herds in Denmark, but Figure 3.11 illustrates the development in the prevalence of non-dairy herds that had at least one antibody positive sample in the last, up to 8, samples from the herd in the national surveillance testing scheme between 2007 and 2012 for 7 categories of non-dairy herds and across all of the herds (national prevalence). Table 3.2 provides criteria for each of the categories together with descriptive statistics of the categories and prevalence estimates in June 2012.

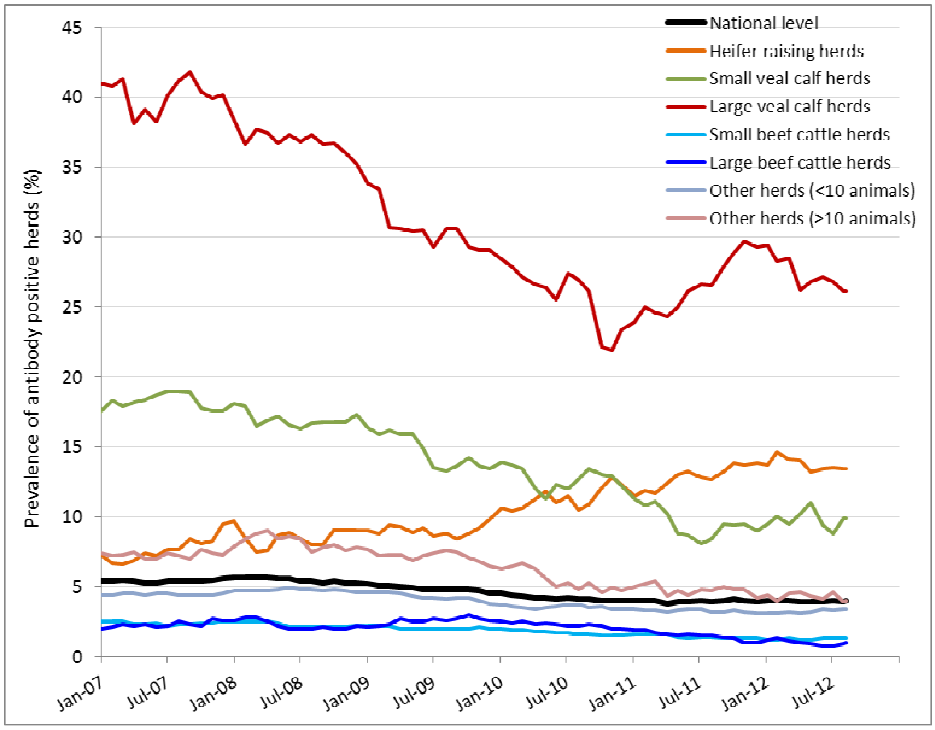

Figure 3.11 The development in the prevalence of antibody positive non-dairy herds in Denmark according to the national surveillance testing scheme from January 2007 to August 2012 (Source: Knowledge Centre for Agriculture, Cattle, Skejby). An explanation of the categories is provided in Table 3.2.

Table 3.2 The distribution of *S.* Dublin antibody positive herds in defined categories of non-milk producing herds in Denmark on 27th June, 2012.

Category (Category definition)	Number of herds (mean;median #animals per year) *(mean;median #animals slaughtered over the last 12 months)*	Percent herds with *S.* Dublin antibody positive samples[a]	Percent herds with unknown status due to too few samples[b]
'Heifer raising facilities' (High proportion of heifers meaning more than 60% dairy-breed and cross-breed heifers out of more than 30 animal years)	764 (137;112) *(8;3)*	13.2%	41.6%
'Small veal calf herds' (Deliver 20 to 100 bull calves to slaughter per year; more than 80% dairy-breed and cross-breed animals)	422 (73;60) *(57;53)*	8.8%	14.0%
'Large veal calf herds' (Deliver more than 100 bull calves to slaughter per year; more than 80% dairy-breed and cross-breed animals)	363 (312;220) *(385;252)*	27.1%	3.6%
'Small beef cattle herds' (Between 10 and 50 animal years; a minimum of 20% cows; beef breeds and cross-breeds constitute a minimum of 80% of all animals per year)	3.937 (23;21) *(7;6)*	1.3%	11.9%
'Large beef cattle herds' (More than 50 animal years; a minimum of 20% cows; beef breeds and cross-breeds constitute a minimum of 80% of all animals per year)	1.025 (97;74) *(33;24)*	0.8%	5.0%
'Other herds < 10 animals per year' (Herds that do not fit into the above categories and have less than 10 animals averaged over the year)	5.430 (4;4) *(2;1)*	3.4%	29.8%
'Other herds ≥10 animals per year' (Herds that do not fit into the above categories and had 10 or more animals averaged over the year)	1.416 (45;25) *(18;9)*	4.7%	24.2%
Total	13.358	4.0%	21.5%

Source: www.kvaegvet.dk, accessed on the 27th June 2012.

[a] Percent test positive herds out of all of those herds that had enough samples collected to be classified according to the legislation for the national surveillance programme for *S.* Dublin, e.g. 8 samples are requested to be able to determine a test status for herds with 10 or more animals.

[b] Percentage of herds that did not have a sufficient number of samples collected and therefore were used for the estimation of per cent antibody positive herds.

Within-herd prevalence in non-dairy herds

In the two studies mentioned above based on the sampling of 71 Danish specialised veal producers, the within-herd prevalence (i.e. the animal level prevalence) was also estimated. Again, the numbers of faecal shedders most likely underestimate the true number of animals that carry *S*. Dublin and other serotypes in faeces at slaughter, given that the Se of the faecal culture is as low as estimated in the Bayesian analysis study (Nielsen et al., 2011 **Paper V**). However, those estimates were not available at the time when Nielsen et al. (2011 **Paper IV**) was published, so an Se of 80% was used for the faecal culture based on a previous study of spiked samples with low concentrations of the *S*. Dublin bacteria in cattle faeces (Baggesen et al., 2007). An Sp of 99.5% was used to allow for potential cross-contaminations of samples either at the abattoir, in the laboratory or by the erroneous identification of samples or recordings. The animal level AP of 1.5% was adjusted to a TP estimate of 1.3% infectious animals across all of the herds. The TP of *S*. Dublin shedding cattle coming from the 12 culture-positive herds varied between 4.8% and 24.5% (Nielsen et al., 2011 **Paper IV**). For a comparison the within-infected herd animal level estimates from the Bayesian study varied between 21 and 49% infected cattle, i.e. 2-4 times higher. It is difficult to say exactly what the underlying detected condition was in that analysis, but a potential interpretation is that this is the percentage of potential shedders in the infected veal calf herds (Nielsen et al., 2011 **Paper V**). To the authors' knowledge, this was the first study to provide presumably unbiased estimates of within-herd prevalence of *S*. Dublin in veal calf herds.

In a study of 93 abattoirs in the UK in 2003, seven different *Salmonella* spp. were found in 36/2553 (1.4%) faecal samples collected from cattle slaughtered at <30 months old. *S*. Dublin was found in eight (22.2%) and *S*. Typhimurium in 10 (27.8%) of the 36 positive samples (Milnes, 2008). An older study of apparently healthy veal calves in the UK found 31/720 (4.3%) animals tested positive for *Salmonella*. Twenty-three (74%) of these were *S*. Dublin. However, only eight (1.1%) of the tested animals had *Salmonella* in the intestinal contents. The remaining 15 were found culture positive in internal organs, lymph nodes or carcass surfaces (Nazer and Osborne, 1976).

Thus, the bacteriological input to cattle abattoirs from the veal calf production in Denmark appears to be either relatively low or difficult to detect. This corresponds well to the low numbers of *Salmonella* bacteria being detected in the Danish fresh meat surveillance at the slaughterhouse (Figure 3.12). This probably also contributes to the fact that in the most recent annual report on zoonoses in Denmark, only 6 of 42 human cases were attributed to the consumption of domestic beef (Anonymous, 2012a). The studies of veal calf herds suggested that the significant clustering of *S*. Dublin shedders or potential shedders in test positive herds provide options for further reductions in the input to the abattoir or handling of the risk from these herds either in the herds or at the abattoir.

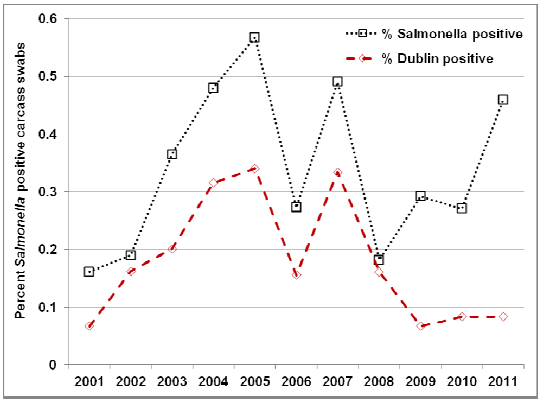

Figure 3.12 The annual proportions of *Salmonella* positive and *S*. Dublin positive carcass swab samples out of all of the tested carcass swabs from the fresh meat surveillance at Danish abattoirs between 2001 and 2011. The testing procedure was changed in 2011. (Source: Danish Zoonosis Centre, 2012).

Chapter 4
Risk factors

Risk factors for introduction of *S*. Dublin to dairy herds

Risk factors for an introduction of *S*. Dublin to cattle herds have previously been investigated in a Dutch cohort study. Fewer purchases of animals and less contact to other herds (i.e. more closed herds) were found to lower the risk of an introduction of the infection (van Schaik et al., 2002). Purchase of cattle has also been found to be an important risk factor for the presence of *S*. Dublin in dairy herds (Vaessen et al., 1998). However, both of these studies were fairly small, resulting in uncertain parameter estimates for the investigated associations.

In 2004 there was a request for new knowledge about the risk factors for an introduction to cattle herds to guide some potential adjustments of the legislation behind the surveillance programme for *S*. Dublin. A study based on the 4 YQs of the surveillance data from 2003 was performed. The objective of the study was to evaluate the risk factors for changing from test negative to test positive based on antibody measurements in BTM. This was considered indicative of herds that become infected. The incidence of new infections varied between 0.013 and 0.054 per herd YQ at risk. The highest incidences occurred in high-prevalence regions such as Himmerland, Southern and South-West Jutland. Other factors that were strongly associated with an increasing probability of becoming infected were the increasing number of test positive cattle herds within a 2 km radius, herd size and the purchase of animals from antibody positive herds (Nielsen et al., 2007 **Paper VI**). This study (among others) encouraged the cattle industry to initiate regional control campaigns in 2007.

In 2009-2010 the *S*. Dublin technical support group led by the Danish Veterinary & Food Administration were working on further adjustments of the legislation. Encouraged by conclusions in the study by Jordan et al. (2008 **Paper XI**), they were looking for potential options to stimulate more control efforts in infected herds and better protection of non-infected herds. One of the questions posed was, how long herds would have to be the subjects of continued targeted control efforts against *S*. Dublin introduction and reinfection after becoming test negative to reach a similar risk as the herds that had not previously been infected. A register-based study was therefore performed using data from all of the Danish dairy herds between 2003 and 2009 (Nielsen and Dohoo, 2012 **Paper VII**). The herds were considered at risk when they had been test negative for at least four consecutive YQs, either at the start of the study period or after a recovery from infection. Survival analysis was performed on a dataset including 6,931 dairy herds with 118,969 YQs at risk, in which 1,523 new infection events occurred. Accounting for seasonal patterns, purchase from test positive cattle herds within the previous 6 months was associated with a markedly higher hazard of *S*. Dublin introduction compared to no purchase and purchase from test negative herds. Increasing local prevalence, herd size and BTM somatic cell counts were also associated with the increasing hazard of new infections. The effect of prior infection was time-dependent, i.e. the hazard of a new infection fell the longer the herd remained test negative. The hazard was markedly higher in herds with prior infections the first year after becoming test negative, and then approached the hazard in herds without known prior infections 2 to 3 years after becoming test negative as illustrated in Figure 4.1 (Nielsen and Dohoo, 2012 **Paper VII**). Apart from being the largest study to date that investigated *S*. Dublin incidence and risk factors for an introduction of the infection to dairy herds, this study is to the authors' knowledge the only study

in which time-varying effects of risk factors for *S*. Dublin at herd level have been investigated. Finally, the study provided essential information about the importance of a continued focus on internal and external biosecurity to avoid a re-infection or a new infection for up to 3 years in herds that have controlled *S*. Dublin and reached a test negative herd status. This is important because farmers may otherwise have a tendency to stop the control efforts as soon as the herd becomes test negative.

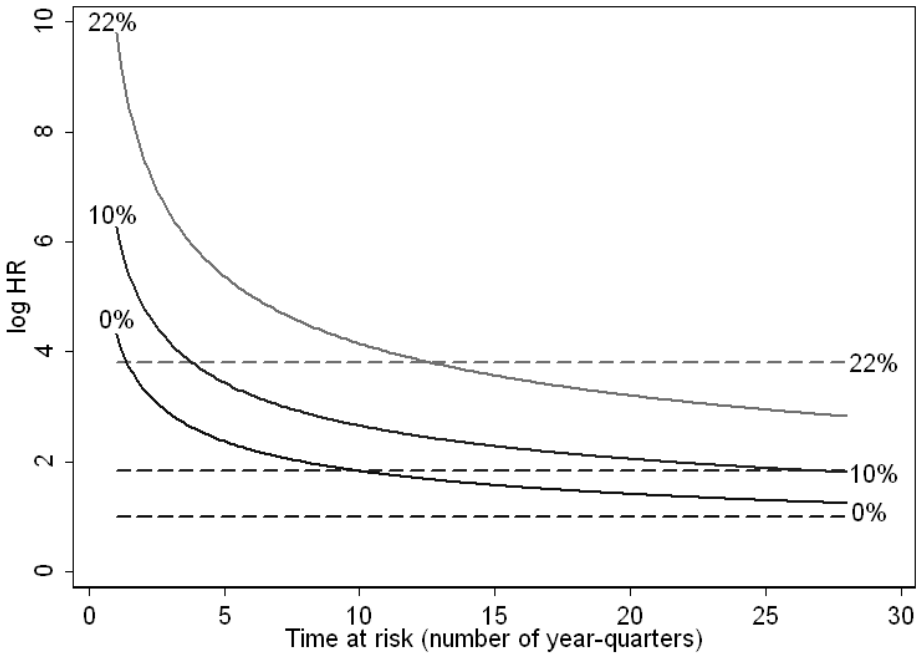

Figure 4.1 The model-predicted log hazard ratio (log HR) for the effect of prior infection on the hazard of *S*. Dublin introduction to dairy herds as a function of time at risk (number of year-quarters at risk) at three different local prevalence levels (0%, 10% and 22%). Horizontal dashed lines indicate the baseline hazard (no prior infection) for the given local prevalence group.

The effect of purchase from test positive cattle herds can be explained by carrier cattle posing a risk of transmitting bacteria when moved from their herd of origin. The stress of transportation, change of environment and change of feed may lead to faecal shedding of bacteria with no concurrent clinical signs (Mattila et al., 1988; Wray et al., 1989; Wray et al., 1991). The spatial associations between cattle herds indicating a spread of *S*. Dublin between the neighbouring herds in local areas of varying sizes have been investigated further and confirmed in two other Danish studies (Ersbøll and Nielsen, 2008; Ersbøll and Nielsen, 2011). The range of influence varied between 1.5 and 8.3 km in 2005, and spatial clusters changed markedly over time from 2003 to 2009 together with reductions in prevalence. The actual transmission pathways between herds remain speculative, because they could not be explored in the register-based studies. Trade was taken into account in the studies by Nielsen et al. (2007 **Paper VI**) and Nielsen and Dohoo (2012 **Paper VII**), so it is likely that the effect of the local prevalence of test positive cattle herds is a proxy for more diffuse transmission pathways such as contact via pastures or contamination of pastures by slurry from infected herds (Taylor and Burrows, 1971). Also water, vehicles and people

may act as mechanical vectors of bacterial transmission (Wray et al., 1991; Visser, 1998; Vaessen et al., 1998; van Schaik et al., 2002; Kirk et al., 2002). Rodents and birds are less likely to spread *S*. Dublin between farms than other serotypes of *Salmonella* (Gibson, 1965; Wray and Davies, 2000), even though this impact of transmission via large flocks of migrating birds has not been investigated. It is recommended to direct future research towards investigations into the impact of environmental infection pathways on new *S*. Dublin infections in cattle herds to ensure the success of the eradication programme.

Persistence of infection and predictors for recovery from *S*. Dublin in dairy herds

Factors preventing persistence (i.e. durations of *S*. Dublin infection lasting longer than approximately one year after its introduction to the herd) is the primary concern in control programmes at herd, regional and national level. Two studies were performed to quantify the effect of risk factors based on register data from the Danish Cattle Database and the national surveillance data. In the first study, the objective was to evaluate the risk factors for changes from test positive to test negative, which was assumed to be indicative of herds recovering from infection between two consecutive YQs in 2003 (Nielsen et al., 2007 **Paper VI**). Recovery probabilities varied between 0.093 and 0.207 per herd YQ at risk. The highest probabilities of recovery were found in regions with a low prevalence such as the islands Funen, Zealand and Bornholm. In total, there were 421 more recovery events than incidence events in Denmark during 2003, which explains the reduction in the national prevalence during the same period. Larger, organic herds consisting of large breeds with close-contact neighbours and a high regional and local prevalence were less likely to recover than conventional herds or herds consisting mainly of Jersey breed, without close-contact neighbours, and a low regional and local prevalence (Nielsen et al., 2007 **Paper VI**). This study provided evidence that the control campaign had to be targeted towards high prevalence regions and herds with specific management challenges such as large herds and organic herds.

The second study utilised the unique opportunity to extract systematically collected repeated BTM antibody measurements from all of the Danish dairy herds during a 10 year period to perform time-to-event analysis of the factors that affect the recovery of an *S*. Dublin infection at herd level. The strength of the study was the large number of herds that could be classified as infected for at least one YQ (n=3,563), the high number of recovery events (n=3,246) and the long duration of the study period (10 years) representing 36,429 YQs at risk of recovery. The average duration of test positivity was approximately 2½ years (9.9 YQs). However, the duration was time-dependent and declined over time. For a comparison, another study based on the same type of register data from the first 4 years of the surveillance programme, estimated that the duration of infection in *S*. Dublin infected dairy herds was best described by an exponential distribution with a mean of 726 days (almost 2 years) (Jordan et al., 2008 **Paper XI**). It may take half a year for BTM antibodies to decline after *S*. Dublin has been cleared from the herd, so an average duration of *S*. Dublin infection of 2 years is currently the best estimate of an expected duration under the circumstances similar to the structure or management in the Danish dairy cattle industry. However, this depends on certain risk factors. There was an increasing tendency towards the persistence of infection when the local prevalence in a 5 km radius increased, herd size became larger and BTM somatic cell counts went above the average national level. Furthermore, herds with organic farming and farms that had purchased cattle from test-positive cattle herds had a lower hazard of recovery (and thus a longer duration of infection) than conventional herds and herds that did not have risky

purchase behaviours. As illustrated in Figure 4.2, herds that were enrolled in a voluntary control programme for paratuberculosis (PTB) had markedly shorter durations and therefore lower risk of remaining persistently infected in high prevalence regions than herds not participating in the PTB programme. There was no difference in the hazards of recovery for the herds enrolled and the herds that were not enrolled in the PTB programme under low prevalence conditions. This suggested that herds enrolled in the PTB programme had a superior biosecurity and this prevented the introductions and reinfections of *S*. Dublin. The potential synergetic effect and cost-effective advantages of multiple-disease control programmes should therefore be investigated.

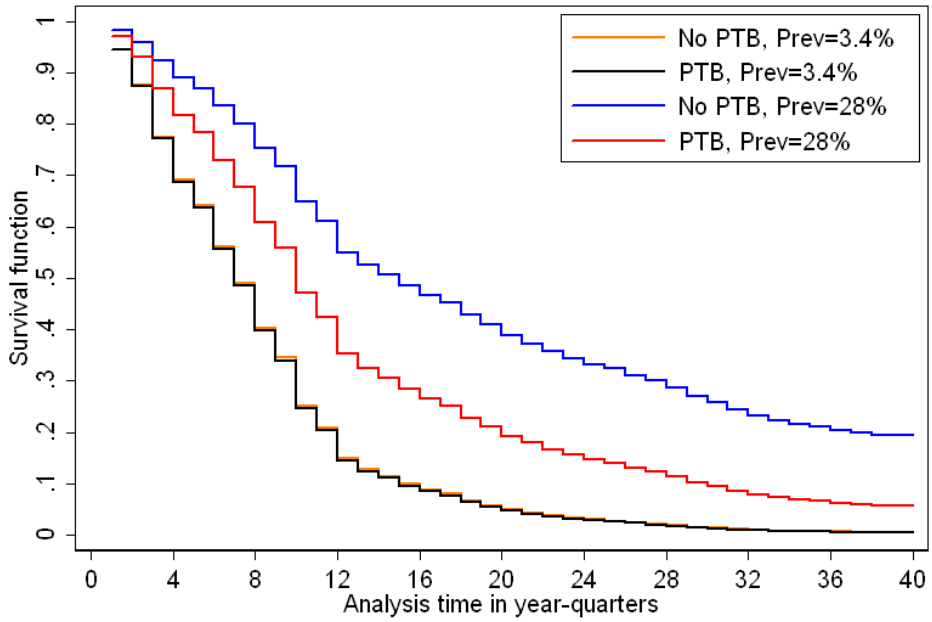

Figure 4.2 Predicted survival curves for recovery from an *S*. Dublin infection in Danish dairy herds between 2002 and 2012 at low (3.4%) and high (28%) local prevalence ('Prev') for herds that did and did not participate in the voluntary paratuberculosis programme(PTB) (Nielsen and Dohoo, 2013 **Paper VIII**).

There was evidence that control of *S*. Dublin was stimulated by centrally organised initiatives. The hazard of recovery from *S.* Dublin increased and remained high in 2002-2004 around the initiation of the surveillance programme (YQ 2 to YQ 11). Another wave of improved hazards of recovery was observed from early 2006 to mid 2007 (YQ 16 to YQ 22). This may have been induced in early 2006 by a change in the surveillance classification programme, which made it possible to reach the desirable 'Level 1' (test-negative) herd classification faster, when controlling the infection. A third wave of improved hazards of recovery started in YQ 25, approximately half a year after regionally targeted control efforts were initiated, and continued throughout 2008 and 2009 up to around YQ 32 corresponding to the first YQ of 2010 (Figure 4.3). After that the hazard of recovery appeared to decrease distinctly, which corresponds well to the stagnation in prevalence observed in the surveillance programme during 2010 and 2011 (see Chapter 6) (Nielsen and Dohoo, 2013 **Paper VIII**). To the authors' knowledge, this is the only large scale study available that systematically evaluated the duration, the risk factors of persistence and the time-dependency of the risk factors of *S*. Dublin in dairy herds.

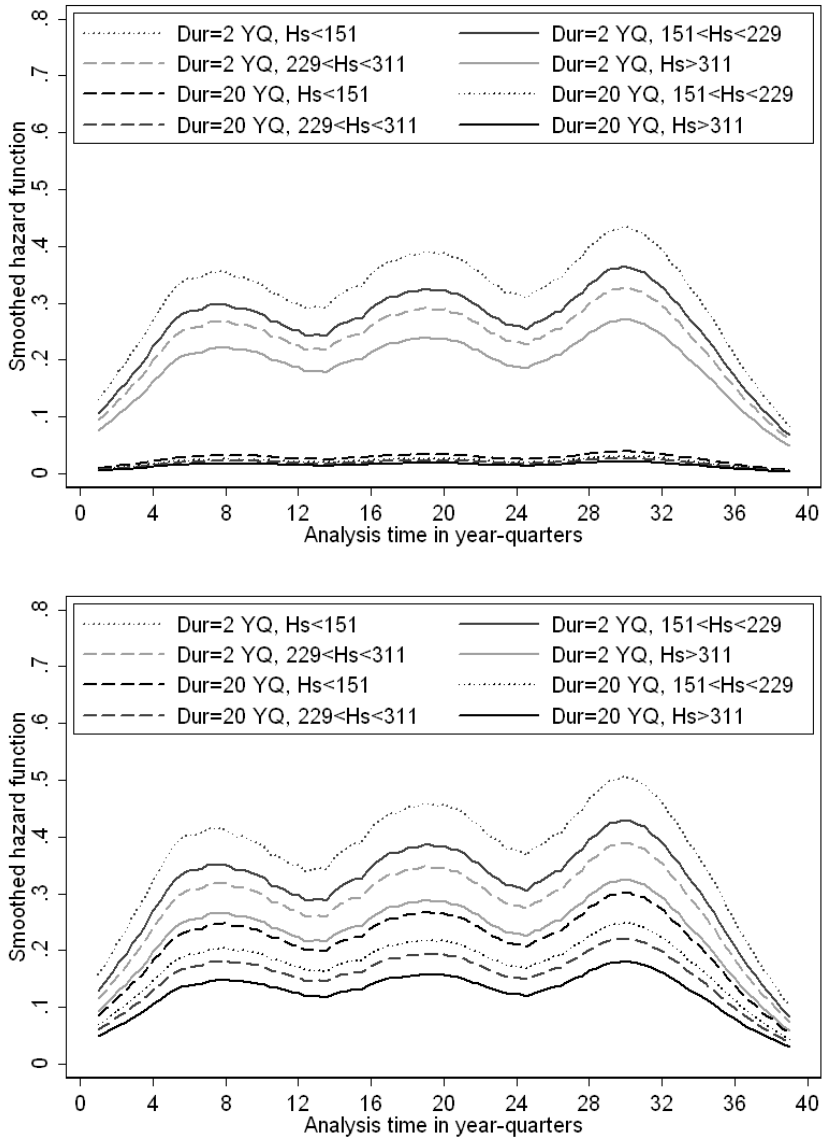

Figure 4.3 Smoothed hazard functions for the recovery from an *S*. Dublin infection in Danish dairy herds with four herd sizes and short (2 YQs) and long durations (20 YQs) at risk between 2002 and 2012 at a low local prevalence (3.4%). These herds were assumed to be conventional, to not have purchased cattle and to participate in the voluntary paratuberculosis programme. The top graph shows the hazard under the conditions early in the study period, i.e. in the third YQ of 2004, and the bottom graph shows the hazard matching the conditions late in the study period, i.e. in the third YQ of 2009 (Nielsen and Dohoo, 2013 **Paper VIII**).

Internal biosecurity routines of relevance for the persistence of infection in cattle herds could not be investigated in these studies, because they were based on the register data from the Danish Cattle Database. However, such factors are important elements of management challenges during the spread and control of *S*. Dublin, including cleaning routines, housing facilities and barn sectioning, calving management, and feeding practices, handling and administration of colostrum,

control of other diseases and rodent control (Tablante and Lane, 1989; Hardman et al., 1991; Steinbach et al., 1997; Veling, 2004; Nielsen et al., 2012b).

Comparing with studies from other countries, a Swedish study showed that 4 of 84 *S*. Dublin and 2 of 21 *S*. Typhimurium infected cattle herds, which were followed over time, were difficult to clean up and therefore were under restrictions with *Salmonella* diagnoses for more than 600 days after the first isolations (Boqvist and Vågsholm, 2005). However, the duration of infection in Swedish herds is difficult to compare to the Danish situation, due to very strict law enforcement of control actions aiming at the elimination of *Salmonella* from infected herds in Sweden. A study from the Netherlands suggested that approximately half of the dairy herds that experienced an outbreak of *S*. Dublin became persistently infected, and the probability that the infection became persistent in the herd depended on how well its transmission could be limited early in the outbreak (Veling, 2004). However, the herds were not followed for more than 2 years, so it was not possible to systematically evaluate an expected duration and the factors affecting the duration of *S*. Dublin upon a new introduction of the infection in cattle herds based on that study.

Risk factors for *S*. Dublin in non-dairy herds

As mentioned earlier, non-dairy herds are part of a very heterogeneous group both with regard to production, herd size, breeds, housing, grassing practices and management in general. Published studies of the risk factors for *S*. Dublin in non-dairy herds mostly concern large beef and veal calf herds, and even for those groups informative studies are scarce or non-representative for Danish conditions (e.g. often concern other serotypes than *S*. Dublin).

Risk factors for *Salmonella spp.* in cattle herds generally include hygienic factors in the herds e.g. flies in pens (Vanselow et al., 2007), contact with poultry manure or wild bird manure, outdoor calving, herd size and herd expansions (Warnick et al., 2001). Hygiene and contacts at markets and in vehicles are also important risk factors before slaughter (Wray et al., 1991). In Denmark, specialised veal-producers purchase bull calves from dairy herds around the age of 2 to 4 weeks and rear these animals until slaughter. Many veal calves are slaughtered before the age of 12 months. They may be infected with *Salmonella* in the herd of origin, the rearing herd or during transportation or lairage at the abattoir, even though generally transportation and lairage times are short in Denmark.

A study was performed to investigate the risk factors for *S*. Dublin in veal calves under Danish conditions. The study was based on the faecal culture of veal calves delivered to slaughter from 71 randomly selected specialised veal ("dairy-beef") producers that were expected to deliver more than 100 calves to slaughter per year. *S*. Dublin bacteria were isolated from 12 herds, and logistic regression analysis showed that the herds that had purchased animals from test-positive dairy herds within the year prior to the sampling period had a higher risk of delivering *Salmonella*-shedding calves to slaughter than herds that had only purchased animals from test negative dairy herds. The number of purchases from other herds is naturally correlated with the herd size. However, some of the large Danish specialised veal producers did not purchase from test positive dairy herds, and also had zero test positive samples at slaughter. This emphasises the importance of control efforts in the dairy herds to ensure animal health in veal calf herds and food safety for consumers (Nielsen et al., 2011 **Paper IV**).

Duration of infection and its persistence in individual cattle

The duration of infection in individual animals has to be seen in relation to the course of infection, i.e. whether the animal is only transiently infected or whether it becomes a persistent carrier of the infection (Robertsson, 1984; Smith et al., 1989; House et al., 1993; Nielsen et al., 2004a). Experimentally, calves inoculated with *S*. Dublin typically shed bacteria in faeces more or less continuously for 2 weeks, then intermittently for an undefined period of time. However, experimental studies are often based on large inoculation dosages which make them less relevant for the epidemiology and control of *S*. Dublin under field conditions (Wray and Sojka, 1981). Therefore a cohort field study was performed to investigate bacteriological and serological patterns in young calves in four endemically infected Danish dairy herds. The sample collection was organised over a period of four months, so that all of the calves that were born in the study period (n=88) were sampled every 3 or 4 days for the first 4 weeks after birth and then once per week. All of the neighbouring calves in the same barn areas were sampled once per week. All of the calves were below 180 days old. In total 181 calves were sampled in the study period. The duration of *S*. Dublin infectiousness in the 19 calves, that were culture positive at least once, was estimated from bacteriological culture of their faecal samples. The duration of infectiousness was estimated to be on average 17 days (median=10). However, the range was wide (3 to 68 days). Isolations of *S.* Dublin bacteria were made in all ages of the calves including calves that were a few days old. In the same study, the time to seroconversion after the onset of shedding was estimated from repeated antibody measurements in serum samples to be on average 36 days (range 11-67 days)(Nielsen et al., 2007 **Paper III**). Thus, there appeared to be coherence between the results from experimental studies and this field study on important elements of transmission dynamics. However, persistent infection in individual animals could not be investigated in this study, as carriers can be infected and/or infectious for months or years (Richardson, 1973; House et al., 1993). They are therefore potentially important for the persistency of infection within herds. This will be discussed further in Chapters 5 and 7.

Chapter 5
Infection dynamics

Infection dynamics under different farming and housing conditions can advantageously be studied using simulation models. These can also be used to study the potential effects of infections and control strategies against the infection both at within-herd and between-herd level (Nielsen et al., 2011). As stated by Premashthira et al. (2011): 'Updated observational information is very important for epidemiological modeling'. The requirements of an effective model are that (i) it should behave in a biologically and mathematically reasonable way, (ii) it must be sensitive to important factors and insensitive to unimportant factors, (iii) its mechanisms should be intuitively acceptable and (iv) it should mimic real-life situations.' Apart from this, the choice and specification of simulation models depend on the purpose, type of infection, access to data for input parameters and probably also personal preferences.

Simulation modelling of transmission between dairy herds

Few studies have been published that model transmission of *S*. Dublin between cattle herds in larger geographical areas. Bergevoet et al. (2009) used a stochastic Monte Carlo simulation model approach to assess the epidemiologic and economic effects of control measures of *Salmonella* in the dairy sector in the Netherlands. Their model covered both *S*. Dublin and *S*. Typhimurium; the two most dominant serotypes in the Netherlands. The epidemiological module was based on the modelling of four herd infection states (i.e. 'susceptible', 'infectious', 'carrier' (persistently infected with carrier animals present in the herd) and 'recovered') and the probability of state transitions that were determined by known risk factors such as hygiene level, purchase of animals, barn sectioning, colostrum handling, introduction of manure from other herds, liver fluke infestation in the herd, neighbourhood contacts and contacts with other species. Monthly time steps were used in the simulation of different scenarios. The results of the simulated control scenarios are discussed and compared to Danish studies and experiences in Chapter 7. Whereas the Dutch dairy industry might resemble the Danish, the dairy herds are generally smaller, the number of herds is higher and they are distributed over a smaller area leading to a higher risk of spread between neighbouring herds. This may change the dynamics enough that the most optimal control strategy in Denmark might differ to some extend from that found by Bergevoet et al. (2009). Furthermore, the effect of using systematically collected surveillance or herd classification data while taking into account the unavoidable misclassifications of such data were not investigated in that study.

Another stochastic Monte Carlo simulation model based on similar principles, but specifying four different infection states for the herds (i.e. 'susceptible', 'clinical', 'chronic' and 'flareup' of infection) was used to evaluate the consequences of the introduction of *S*. Dublin to New Zealand, which was assumed to be free from the infection at the time of the study (Sanson and Thornton, 1997). The main between-herd transmission routes considered were direct or indirect contact of cattle via farm-to-farm movements, truck contamination and sale yards. The outcomes of interest were the surveillance system sensitivity, the time to detection of the first case and the epidemic size after 52 and 78 weeks. Two surveillance scenarios were investigated: 1) the surveillance programme in place; and 2) an alternative reduced surveillance scenario based on reduced governmental funding of diagnostic procedures, which would lead to fewer tests being performed upon clinical suspicion. The surveillance in place was a passive surveillance system based on Each herd that became infected upon exposure would go through this cycle in the modelled scenarios. In this model no differentiation was made between the herds with clinical outbreaks and the herds

that became infected without any obvious clinical signs associated with the infection. However, natural variation in within-herd prevalence was included in the input parameters, and this is assumed to cover the difference in infectiousness between clinically affected and subclinical *S*. Dublin infections at a herd level.

bacteriological culture of clinical suspicions possibly supplemented with a slaughterhouse sampling scheme that was not specified clearly in the paper (Sanson and Thornton, 1997). Whereas that study might be relevant in the future for Denmark, if *S*. Dublin gets eradicated from Denmark, it was not that relevant in 2006 when decisions were being made about future control strategies at a national level. At that time there were still around 16% test positive dairy herds in the country, and the prevalence seemed to have stabilised at that level.

To support future decisions about where to focus actions in the control programme for *S*. Dublin in Danish cattle herds, a simulation model was developed at the International EpiLab in Denmark in 2006. Register data and knowledge from epidemiological studies and field data were used to model the effect on herd level prevalence of different control scenarios for *S*. Dublin on a national level over a 10-year period (Jordan et al., 2008 **Paper XI**). Rather than going with the frequently used 'SIR' infection dynamics type of model that requires the specification of transmission parameters, this model was constructed as a 'virtual hierarchy' model which arranged animals, herds and geographic regions in the Danish dairy cattle industry as a hierarchy of objects in computer memory. Superimposed on all of the objects were an infection–recovery cycle, a control programme, test results and animal movements used for herd classification in the Danish surveillance programme. The infection-recovery cycle included a true negative period, which corresponds to the susceptible state in the models described above, a dissemination period, which was not included in the other models, an antibody lag period and a true positive period that correspond to the infectious states in the other two models described above, and an antibody fall period which corresponds to the recovered period in the model by Bergevoet el al. (2009) (Figure 5.1).

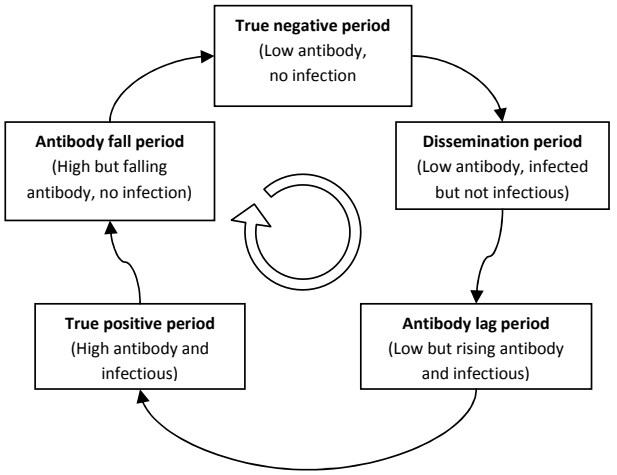

Figure 5.1 Diagram of the infection-recovery cycle of *S*. Dublin in Danish dairy cattle herds used to model the temporal changes in surveillance status of herds and their true infection status (Jordan et al., 2008 **Paper XI**).

Distributions determined the duration of each of these periods to represent natural variability in the infection cycle. A total of 7,000 dairy herds in seven regions of Denmark were assigned a true infection status and a surveillance test status in each daily time step, and 1,000 iterations were run to represent the stochasticity of the process. Assumptions regarding the within-herd prevalence, the infectiousness of individual animals, the within-herd dissemination of infection, the recovery etc. were used to mimic elements of importance for the spread of infection between herds. An external environmental probability component was added to allow for new infections to occur without the movement of cattle between herds, as is seen in real life. Herds were assigned closed, conservative or indiscriminate purchase policies based on the distributions estimated from the data from the Danish Cattle Database. Five control scenarios (i.e. regional restriction of animal movement, enhanced external biosecurity in all herds, more frequent herd testing in the surveillance programme, enhanced control at herd level and a composite scenario of all scenarios) were tested in the model. These scenarios and the results of the model simulations are presented and discussed in Chapter 7 about the control of *S*. Dublin.

The approach used in the study was a novel approach to the simulation of infection dynamics, where animals, herds, and geographic regions in a national livestock industry were arranged as a hierarchy of objects in computer memory. In principle one would be able to follow a specific herd through the simulations. This made the modelling approach intuitive and easy to explain to farmers and decision makers. It was also easier to discuss the input parameters with experts that have field experience. Together these qualities make the results more likely to be used by decision makers. The results of the study by Jordan et al. (2008 **Paper XI**) were specifically used by the working group of the *S*. Dublin surveillance and control programme led by the Danish Veterinary and Food Administration in their action plan report to the Minister of Food, Agriculture & Fisheries in 2009 (Anonymous, 2009a).

Infection dynamics within dairy herds

Within-herd transmission of *S*. Dublin varies considerably between herds, and depends on many environmental factors such as the housing structure and the separation of different barn sections, stocking density, group sizes, movement of animals through the herd including the mixing of animals from different age groups, and management related to hygiene, feeding, calving procedures and colostrum management which affect exposure and/or susceptibility of the host (Jensen et al., 2004; Veling, 2004; Boqvist and Vågsholm, 2005; Nielsen et al., 2012b). Transmission between individually housed calves has been suggested to primarily occur via passive horizontal transfer between pens on utensils and barn equipment contaminated with infected faeces (Hardman et al., 1991). The splashing of a few drops of highly *Salmonella* contaminated material into, for instance, feeding or water buckets may expose a susceptible host (Bemis et al., 2007). Furthermore, horizontal spread within a herd might occur via mice that have been infected by cattle in the herd (Tablante and Lane, 1989). Survival in the environment is also thought to be an important element in within-herd spread (Plym-Forshell and Ekesbo, 1996). However, it is essentially impossible to investigate the importance of each of these factors and the effect of changing each factor as part of a control strategy in a controlled longitudinal field study, due to the financial restrictions which limits the number of herds that can be included in such studies. Furthermore, a lack of compliance with study protocols in observational studies in real life herds is a common problem. However, until recently there were no simulation models available specifically for *S.* Dublin within-herd epidemiology.

The 'Dublin-Simherd' model

Therefore an age-structured stochastic, mechanistic and dynamic simulation model of *S*. Dublin in dairy herds was developed that aimed to facilitate an investigation into factors leading to the transmission of infection (or reducing the spread of infection) and the effects of the infection in dairy herds. Another aim was that the model could be used as a decision support tool to guide farmers who want to control *S*. Dublin, so it had to be possible to simulate herd specific scenarios.

The model was constructed as an object-based addition to an existing dairy herd simulation model, Simherd. The new *S*. Dublin module, "Dublin-Simherd", incorporated six age groups (neonatal, preweaned calves, weaned calves, growing heifers, breeding heifers and cows) and five infection states (susceptible, acutely infected, carrier, super shedder and resistant). Animals were born susceptible, and if exposed could become acutely infected. Distributions determined the morbidity, mortality, treatment probabilities and effects and the duration of infection in acutely infected animals. Probability inputs determined whether after an acute infection, the animal became a super shedder (rare event), carrier (more commonly) or resistant (most commonly). Super shedders were defined as animals that remained as infectious as animals in the acute state for a prolonged period defined by a distribution. Some might define this as an active carrier rather than a super shedder. However, in the literature, cattle suggested to be 'active carriers' or simply 'carriers' were found to shed 10 to 10^5 CFU/g faeces (Sojka et al., 1974; Christensen, 2005), which is less than acutely infected animals would typically shed (Wray and Sojka, 1981; Christensen, 2005). Veling et al. (2000) defined active carriers as cattle with at least three successive positive faecal cultures with a sampling interval of at least 14 days, but they did not specify whether the concentration of bacteria in the faeces from the 'active carriers' was similar to that of an acutely infected animal. In the Dublin-Simherd model, we included the super shedder state as a continuation of the acute state most commonly in clinically ill animals, to explore whether such a state is necessary to be able to mimic endemic herd infections observed in the field. Based on literature (Robertsson, 1984; Steinbach et al., 1997; Veling et al., 2000) the probability of becoming a super shedder was set low (0.5%), if the animal did not become clinically ill, and moderately high (27%), if the animal had become clinically ill from the infection. The carrier state was defined as a prolonged infected with 100 times lower probability of faecal excretion in the Dublin-Simherd model (Nielsen et al., 2012 **Paper IX**).

The model kept track of each individual animal in the virtual herd in weekly time steps. Changes in the cattle population and infection dynamics within the herd were mimicked for a period of 10 years with a variation in the following parameters: 1) population sizes of each of the six age-groups; 2) *S*. Dublin incidence and the number of animals in each infection state; and 3) *S*. Dublin related morbidity and mortality in the acutely infected animals. The effects of introducing one infectious breeding heifer on the risk of a spread of *S*. Dublin within the herd and on the duration of infection were estimated through 1000 simulation iterations for 48 scenarios. The scenarios covered all combinations of three herd sizes (70, 200 and 400 cows), four hygiene levels indicating infectious contact parameters, and four herd susceptibility levels indicating different susceptibility parameters for the individual animals in each of the six age groups in the herd (Nielsen et al., 2012 **Paper IX**). An example of the resulting infection dynamics in two of the age groups in one of the iterations with a spread of infection is illustrated in Figure 5.2. The patterns varied between each of the iterations.

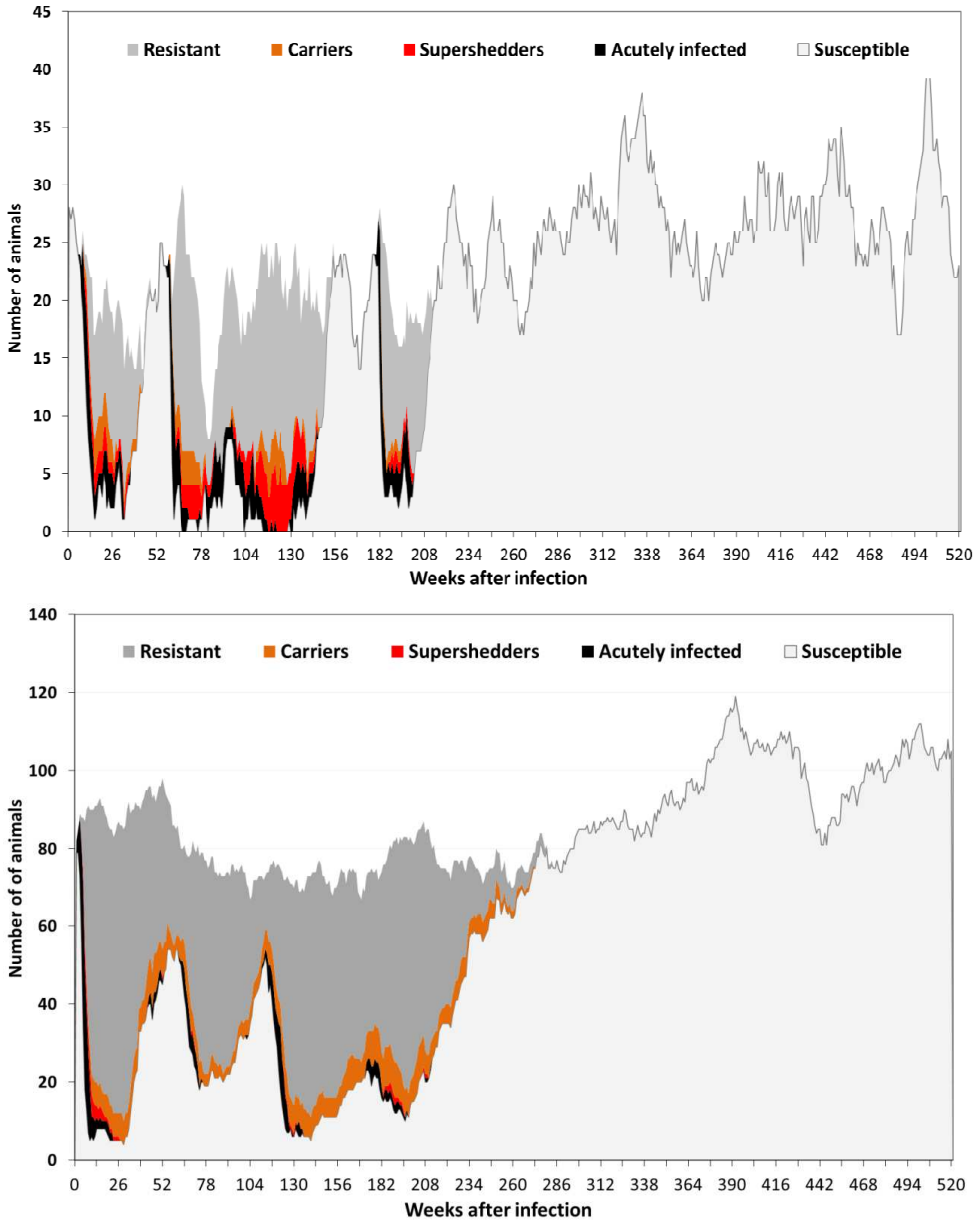

Figure 5.2 An example of the infection dynamics over a 10-year period in two of the six age groups of cattle (top graph: weaned calves 8-26 weeks old and bottom graph: breeding heifers +1 year old) in a simulated Danish dairy herd with 200 cows, a herd susceptibility level 2 and an average hygiene level (default scenario). The infection came into the breeding heifer age group with the purchased infectious heifer in week 1, and into the weaned calf section in week 7. This herd had several epidemics of *S*. Dublin with a total of 131 diseased and 39 dead animals due to *S*. Dublin over the 5.7-year period that this herd was infected (Nielsen et al., 2012 **Paper IX**).

The simulation results showed that the hygiene level was highly influential on the probability that the infection would spread within the herd, duration of infection and epidemic size. The herd susceptibility level was also influential, but not likely to provide sufficient prevention and control of an infection on its own. In addition, herd susceptibility is a factor that is more difficult to change markedly through daily management unless an effective vaccination is available. The use of a vaccination to control *S*. Dublin is discussed in Chapter 7. Herd size did not affect the probability of a spread of infection upon exposure, but the larger the herd, the greater the importance emphasised on management and housing that optimised hygiene for a reduced susceptibility of the cattle, to shorten durations of infection in the herd and to increase the probability of extinction.

Sensitivity analyses of 24 alternative scenarios showed that a super shedder state was not essential to mimic real life infection dynamics, which may indicate that this state does not exist or only very rarely occurs. However, a persistent carrier state was required to mimic the infection dynamics and persistence patterns known from field studies (Nielsen et al., 2012 **Paper IX**). Transmission from adult carriers to calves has previously been described (Richardson, 1973). This is important around the time of calving when contact between a carrier and a calf is most intense. In this situation the carriers are subject to stress, which may lead to reactivation of a latent infection or an increased excretion of bacteria from the carriers (Spier et al., 1991; Kehrli et al., 1999). Also, calves are highly susceptible just after birth (Fisher et al., 1976). Carriers not only pose a risk to their own calves in the calving environment, if measures are not taken to avoid cross contamination to the next calving cows and their calves, these may also become infected. Furthermore, excretion from carriers contaminates the environment both indoors in barns and on pasture. However, even without carriers, infection can remain persistent in a herd as long as there is enough entry of susceptible animals and/or sufficient environmental contamination (Nielsen et al., 2007 **Paper III**).

The Dublin-Simherd model differs from previously published simulation models by its object-based, mechanistic nature that is based on direct contact structures between individual cattle in the simulated barn sections and by its incorporation of an indirect feedback mechanism in the herd dynamics. Furthermore, it takes into account other factors in the herd that might affect infection dynamics such as reproductive performance, other diseases, feeding and culling strategies (Østergaard et al., 2000; Østergaard et al., 2005). This intuitive model construction is an advantage when attempting to explain the model to farmers or cattle advisors. To the authors' knowledge this is the only existing model that includes infection, population dynamics and feedback mechanisms of *S*. Dublin to this level of detail. Other theoretical simulation models of *Salmonella* in cattle herds described in the literature were not serotype-specific, but some investigated features that could be related to serotype-specificity. These models were theoretical, mathematical SIR-models estimating and comparing the basic reproduction number, R_0, between different age-groups and scenarios (Xiao et al., 2005; Xiao et al., 2007; Lanzas et al., 2008). The R_0 essentially describes the contagiousness of the bacteria as the expected average number of new infectious cases caused by a single case during its period of infectiousness in a completely susceptible population. R_0 was estimated to vary between 1 and 2.5 based on field data in four Danish dairy herds (Nielsen et al., 2007 **Paper III**). Similar R_0 estimates were found by Lanzas et al. (2008), who also constructed their model based on experience from field outbreaks of different *Salmonella* serotypes in the USA. If R_0 is above 1, it is assumed to be unlikely that the infection will

die out by chance and that the infection typically becomes endemic. However, R_0 estimates up to 1 to 2.5 are not very high compared to e.g. some viral diseases that spread through large populations, such as measles which has an R_0 above 10. The outbreaks observed in the Dublin-Simherd iterations that lead to within-herd transmission showed patterns that were compatible with the R_0 estimates found in the mentioned studies. Lanzas et al. (2008) found that super shedders were more important drivers of the infection than persistent carriers. The difference between the their study and the conclusions from the Dublin-Simherd study on that matter may be due to the definitions of super shedders and carriers with regard to infectiousness.

The simulation results on the duration of infection from the Dublin-Simherd study can be compared to the time to recovery analysis on observational data presented by Nielsen and Dohoo (2013 **Paper VIII**). The median herd size in the observational study was 229 animals corresponding to approximately 115 cows, and the duration of infection was estimated to be on average 2 years, but varied between 3 months and 10 years. The results should be compared to the results from herds with 70 cows (small herds) and 200 cows (medium sized herds), respectively, in the simulation study by Nielsen et al. (2012 **Paper IX**). In the default scenario with average hygiene and herd susceptibility, the duration in small herds was on average 2.2 years (5^{th}-95^{th} percentile: 3 months to 4.9 years). The duration in medium sized herds was 7.4 years (5^{th}-95^{th} percentile: 2.4 to 10 years). In other words the observational epidemiological study corresponds to the results simulated in the small herds. However, it must be assumed that a large proportion of the infected herds in the observational study performed control actions to try to eradicate *S*. Dublin from the farm encourage by the Danish surveillance and control programmes. Control efforts were not included in the simulation study. Even though this does not validate the model as such, it does provide evidence that the model reflects reality in the Danish dairy sector.

Effects of *S*. Dublin infection in dairy herds

The Dublin-Simherd model was used to investigate the effect of *S*. Dublin on animal health and economics in herds that experienced a spread of *S*. Dublin upon exposure to one purchased infectious breeding heifer (Nielsen et al., 2012 **Paper IX**; Nielsen et al., 2013 **Paper X**). In general, disease and mortality patterns followed epidemic waves in the herds. However, an interesting pattern was seen for acute infections and abortions in adult cattle after the first 2 years of infection in herds with poor hygiene and a high susceptibility level. Repeated infections in young stock lead to a high proportion of resistant adult cattle, which in return lead to a dampening effect on acute infections in adults and thus relatively fewer associated abortions (Nielsen et al., 2012 **Paper IX**). This phenomenon of resistance among adult cows may partly explain why some farmers do not perceive the presence of *S*. Dublin in their herd as a problem. However, as demonstrated in an observational study by Nielsen et al. (2012a) *S*. Dublin infection in lactating cows is associated with fairly large milk yield losses (i.e. up to 3 energy-corrected kilograms of milk (ECM) per day for certain periods after the introduction of the infection in third parity cows). The milk yield losses were present not only during the peak of an outbreak, but also for an extended period after the herds experienced large increases in BTM antibody levels indicative of a spread of infection through the cow barn. The milk estimated yield losses were calibrated and included in the Dublin-Simherd model together with estimates of mortality, morbidity, treatment costs and effects obtained from literature (Nielsen et al., 2012 **Paper IX**; Nielsen et al., 2013 **Paper X**).

New 10-year simulations demonstrated that losses in gross margin were highest the first year after the infection was introduced to the herd, but under some scenarios and herd sizes the economic losses were substantial as long as the infection persisted, sometimes even after 10 years (Nielsen et al., 2013 **Paper X**). Figure 5.3 shows the estimated annual losses in gross margin for three herd sizes and four different management levels. The gross margins were estimated per cow stall, because *S*. Dublin infection could change the population dynamics in some scenarios, which lead to a fluctuating number of cows over the infected period. Annual losses of between 100 and 200 euro per cow stall in a herd with 200 or 400 cow stalls were not uncommon, in some cases up to 10 years after the introduction of an infection. The total losses in gross margin over the first year, after an introduction and spread of *S*. Dublin in a dairy herd with 200 cow stalls, were estimated to be between 8,200 and 56,800 euro (equivalent to 61,500 to 426,000 DKK), and the annual economic losses during the full 10-year period were estimated to be between 1,400 and 34,800 euro (equivalent to 10,500 to 261,000 DKK) depending on the hygiene and management levels. The main drivers of these losses were milk yield losses in cows that were not clinically ill, so these economic losses might go undetected by the farmer, or he may think they are caused by other problems in the herd. A smaller (and not easily defined) part of the losses were attributed to clinical disease (i.e. treatment costs, increased mortality and associated milk yield losses), and an even smaller part was attributed to abortions and mortality alone (Nielsen et al., 2013 **Paper X**).

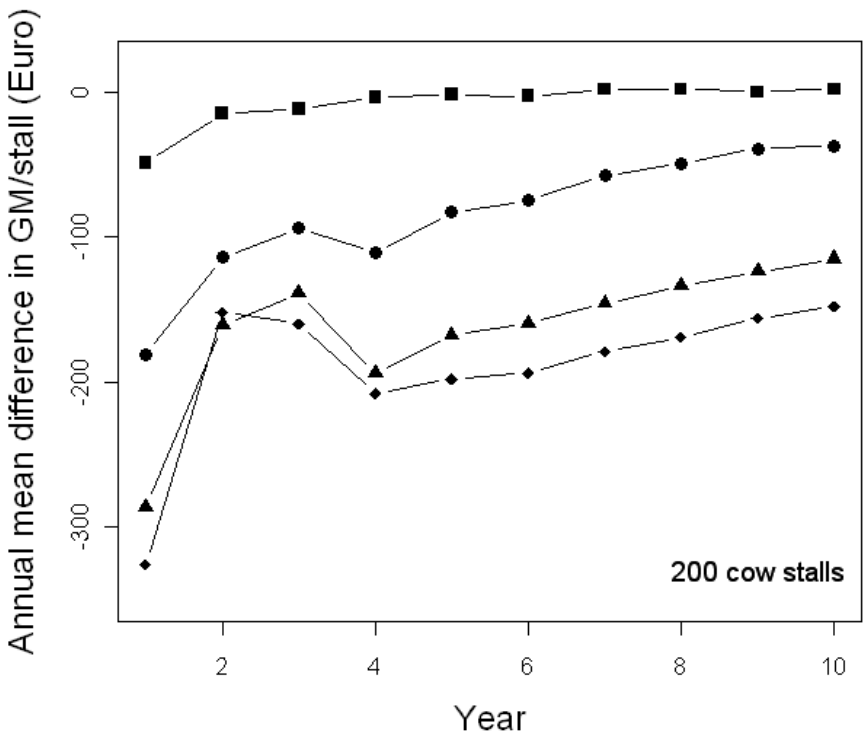

Fig. 5.3 Model-predicted annual differences between infected and non-infected herds in gross margin per cow stall over the 10-year period after an introduction of *S.* Dublin infection into dairy herds with 200 cow stalls and four different management levels. Estimates were derived from 1,000 iterations ■ very good, ● good, ▲ poor and ♦ very poor management(Nielsen et al., 2013 **Paper X**).

Other potential costs and losses that were not included in these calculations were those related to control efforts (which might in turn reduce the losses), diagnostic test costs and costs for advisory services, spread of other diseases or subclinical diseases that might affect the cattle more due to compromised immunity such as paratuberculosis, mastitis or reproductive disorders. Neither were the losses associated with a lower income on the animals sold, because the infected herds would become locked in publicly available test positive surveillance levels, which in Denmark leads to dramatically reduced selling prices on livestock. There were animal health consequences (i.e. mainly clinically ill calves and increased mortality) associated with spread of *S*. Dublin in many of the iterations, but not in all (Nielsen et al., 2012 **Paper IX**). Estimation of the consequences of S. Dublin in infected dairy herds at this level of detail, while adjusting for the other complex feedback mechanisms that commonly occur in dairy herds, has not been done before. The results show that there are ample arguments for initiating control efforts in *S*. Dublin infected herds to avoid long-term losses, even if the required actions involve some investments and other control related costs. Future work is planned to investigate the cost-benefit of different control scenarios in dairy herds, including test-and manage and test-and cull strategies.

Chapter 6
Diagnostic test-strategies and surveillance

This chapter provides a review and discussion of the available literature on test-strategies and surveillance of *S*. Dublin at an animal, herd and national level including the relevant results from papers V and XII-XIV. Recommendations for a design of surveillance programmes in dairy and non-dairy cattle are provided. The chapter is organised so that it starts with herd classifications and surveillance in dairy herds, then moves on to herd classifications and surveillance in non-dairy herds, and ends with studies on animal level testing with a special emphasis on the interpretation of repeated antibody measurements in individual animals.

Herd level classifications and surveillance

In 1998, the Veterinary and Food Administration gathered a group of scientists from different Danish research institutions and the cattle industry to evaluate options for the surveillance of *Salmonella* in cattle herds with a particular focus on *S*. Dublin. This led to two projects funded by the ministry and the Danish Cattle Federation that aimed to evaluate testing procedures for dairy and non-dairy herds (Anonymous, 2001). In the dairy herd project a poor association between high antibody levels in BTM samples measured by a *Salmonella* specific ELISA based on LPS from *S.* Dublin and the probability of isolating the *S.* Dublin-bacteria from faecal samples in the herd was found. However, it was found that herds with low antibody levels in BTM samples were very unlikely to be infected, so it was concluded that the BTM test could be used in dairy herds to certify their 'low risk of *S*. Dublin-infection' (Pedersen, 2003). Different herd level test-strategies were investigated further to decide how to design a surveillance programme for Danish dairy herds with accurate herd classifications at a reasonable cost. Because there was already a surveillance programme in place for BVDv, IBR and leucosis based on BTM sampling in dairy herds and blood sampling in non-dairy herds, it was desirable to use the same samples for *S*. Dublin surveillance.

Herd level test-strategies for dairy herds

Basically, there are two ways to reach a herd level diagnosis: (1) by congregation of diagnostic test results from multiple individual animals in the herd into one output (e.g. testing of *n* randomly selected animals with ELISA and denoting the herd positive, if *x* of the tests are positive), or (2) by analysis of one or more pooled sample(s) that represent all or many of the animals in the herd (e.g. BTM sample, milk filter sample or slurry tank sample(s)). Composite testing strategies might also be relevant, e.g. BTM combined with follow-up testing using blood samples or faecal culture. The herd level sensitivity (HSe) and specificity (HSp) of different simple and composite herd testing strategies for *S*. Dublin have been investigated in three studies (Veling et al., 2001; Veling et al., 2002; Warnick et al., 2006 **Paper XIV**). The results from these studies are summarised in Table 6.1. One interpretation could be that the estimates suggest that if the isolation of bacteria is required for research, tracing or legislative purposes, bacteriological culture of dung pit samples (on average 3.5 samples per herd) or faecal culture of animals with a history of clinical signs of salmonellosis (on average 8 samples per herd) collected 1-3 months after the beginning of the outbreak have the highest HSe (Veling et al., 2002). Testing every single animal in the herd with faecal culture, as it is currently done in Sweden for the free-testing of herds to be released from restrictions due to a *Salmonella* diagnoses, would of course increase the HSe, but would also be an immensely more expensive test strategy, which would therefore only be relevant in countries with

a very low incidence (Boqvist and Vågsholm, 2005; Lewerin et al., 2011). E.g. testing 3 dung pit samples and 8 faecal samples from animals with a recent history of salmonellosis would cost less than 400 euro if each test can be performed for around 30 euro including the sampling, and HSe would be more than 50%. Testing 200 animals with faecal culture would give a HSe close to 100% at a within-herd prevalence of 25% and an individual animal Se of 15%, but the diagnosis would cost more than 6,000 euro assuming that there were the same sampling and testing costs. Pooling of faecal samples would lower the cost, but also lower the Se (Nielsen, 2003). HSe for the detection of infected herds was low for drinking water cultures (approximately five samples per farm) and milk tank filter cultures (two per farm). Even though it was not stated in the study by Veling et al. (2002), the HSp of the culture methods can be assumed to be close to 100%, because false positive bacteriological culture results are rare.

Table 6.1 Herd sensitivity (HSe) and herd specificity (HSp) for different dairy herd test-strategies used for a herd level diagnosis of *S*. Dublin.

Herd testing procedure	**HSe (95%CI)**	**HSp (95%CI)**
Culture of dung-pit samples[b]	44% (33-56%)	NA
Drinking water cultures[b]	5% (0-22%)	NA
Milk tank filter cultures[b]	7% (0-25%)	NA
Faecal culture of animals with current or recent signs of salmonellosis[b]	38% (27-49%)	NA
Serology of all young stock < 1 year old (if one positive, herd considered positive)[b]	96% (92-100%)	NA
Serology of all young stock between 4 to 6 months (if one positive, herd considered positive)[b]	91% (85-97%)	NA
Serology of animals with current or previous signs of salmonellosis[b]	80% (71-88%)	NA
Single BTM, indirect Dutch LPS ELISA at cut-off OD=0.2[a]	54% (44-65%)	98% (96-100%)
Average of previous four BTM ELISA measurements collected over 5 to 12 months (used in the Danish surveillance programme)[c]	95% (93-96%)	96% (92-98%)[d]
Combination of culture of dung-pit samples and faecal culture of animals with current or recent signs of salmonellosis[b]	63% (52-74%)	NA
Combination of BTM ELISA and faecal culture of animals with current or recent signs of salmonellosis[b]	69% (59-80%)	NA
Combination of BTM ELISA and serology of animals with current or previous signs of salmonellosis[b]	91% (85-97%)	NA
Combination of BTM ELISA and serology of all young stock between 4 to 6 months[b]	99% (96-100%)	NA

[a] Veling et al. (2001), [b] Veling et al. (2002), [c] Warnick et al, (2006 **Paper XIV**), [d] estimated at 15% herd level prevalence, NA=not available

The HSe of the test-strategies based on antibody measurements were generally markedly higher than test-strategies based on bacteriological culture, except for ELISA measurements of a single BTM sample collected within 2 to 4 months after the *S*. Dublin outbreak started. A single BTM ELISA had the highest HSe if it was used in herds in which the outbreak started among the adult cattle (HSe=79%, 95%CI: 59-99%), as opposed to herds in which the outbreak started in young stock (HSe=31%, 95%CI: 13-49%) (Veling et al., 2002). The overall most sensitive test strategy was a combination of a single BTM ELISA measurement and the serology of calves between 4-6 months of age. HSp was not evaluated for this combination. However, Veling et al. (2002) argued that the HSp was likely to be high, since the individual serum ELISA has a high Sp (95-100%) for calves in that age group. This was confirmed by other studies (Nielsen and Ersbøll, 2004; Nielsen et al., 2004b). The HSp of a BTM ELISA was estimated to be 98% in Dutch control herds and 100% in Swedish control herds. The test strategy used in the Danish surveillance programme, in which the herd classification is based on the average of four consecutive BTM ELISA measurements, and the deviation in the most recent sample compared to the average of the previous three BTM samples, had slightly lower HSe and similar HSp to the composite BTM and calf serology option as discussed below. However, the cost is much lower and does not require visits to the herd to collect blood samples from the calves.

Surveillance programme for Danish dairy herds

A national surveillance program for *S*. Dublin in all of the cattle herds was initiated in October 2002. At that time, all of the dairy herds had already been tested for antibodies directed against *S.* Dublin-LPS by an indirect ELISA in BTM samples collected every three months throughout 2001 and 2002, so the laboratory was prepared for the challenge. Herds were classified into one of three levels depending on BTM values, contact patterns with other herds and bacteriological culture results from samples submitted by local veterinarians as part of a passive surveillance of salmonellosis as described in Table 6.2. Trade or other types of contact (such as common grazing or contact via markets or shows) were included in the program by a mandatory requirement to record the movements of individual cattle in the Danish Cattle Database.

The levels of all of the herds were made publicly available on the internet (www.glr-chr.dk), but were also included in trade documents that had to follow animals that were moved between herds. The system with the paper documents was stopped, because the use of internet became more widely used. The locking system had a dramatic effect on the trading behaviour of the majority of the dairy sector after the *S*. Dublin-levels became publicly available from October 2002 (Figure 6.1). However, it was not a popular system, so there was a need to document the effect of keeping up the locking system. The scientific argument was documented in a register-based risk factor study included in this thesis (Nielsen et al., 2007 **Paper VI**). It was found that the risk of changing from Level 1 to Level 2 indicative of a new infection in the herd dramatically increased with the number of animals purchased from Level 2 herds in the previous quarter of the year. Odds ratios varied from 3.8-11 compared to no purchase depending on how many animals were purchased (Nielsen et al., 2007 **Paper VI**).

Table 6.2 An overview of herd classification levels in the Danish *S*. Dublin surveillance programme for cattle herds since mid-2010 (Anonymous, 2012a).

Surveillance level	**Official interpretation**	**Criteria**	**Consequences dictated by legislation**
Level 1a	Most likely free from *S*. Dublin-infection	Average ODC%<25 in the last four samples and that had not increased >20 ODC% in the last sample compared to the average of the previous three samples	None – except that if a herd in this level purchases cattle from a herd in Level 2 or unknown it will be locked in Level 2 for at least 3 weeks.
Level 1b	Most likely free from *S*. Dublin-infection	*For dairy herds:* The last 4 BTM < 40 ODC% + 8 blood samples from cattle from 3-24 months old animals all < 50 ODC% F*or non-dairy herds:* The last 8 blood samples from the herd <50 ODC%	None – except that if a herd in this level purchases cattle from a herd in Level 2 or unknown it will be locked in Level 2 for at least 3 weeks.
Level 2	Possibly infected based on too high antibody levels or risky contacts	*For dairy herds:* BTM values above cut-off defined for Level 1a F*or non-dairy herds:* At least one of the last 8 blood samples from the herd ≥50 ODC% For all herd types: Purchase or other types of recorded contact to herds not classified as Level 1a or 1b	Locked here for at least 3 weeks when just moved into this level. Can request extra BTM or blood samples to try to reach Level 1a or 1b based on antibody measurements.
Level 2R	High risk of actively spreading an infection due to high antibody levels	BTM ELISA≥40 ODC% in one of the previous two samples. The Knowledge Centre for Agriculture, Cattle will appoint herds to this level twice annually	Herd put under official veterinary supervision by veterinary authorities, can only send animals to slaughter or to veal calf producers who have signed agreements to be willing to raise calves from herds with a high risk of being *Salmonella*-infected.
Level 3	Confirmed clinical salmonellosis	Clinical signs of salmonellosis + *S*. Dublin confirmed by bacteriological culture	Herd put under official veterinary supervision by veterinary authorities, can only send animals to slaughter at special hygienic slaughter lines.

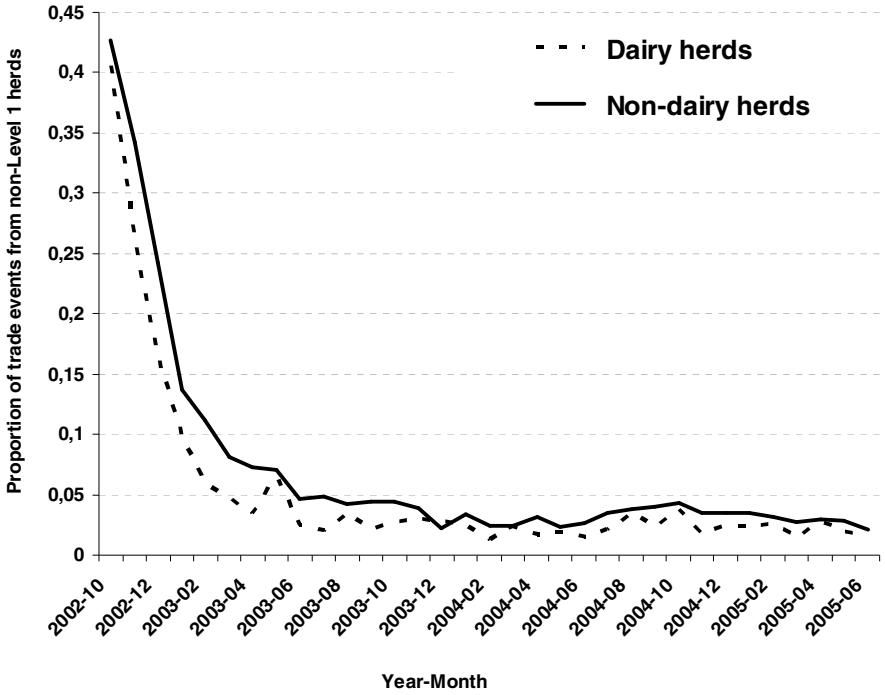

Figure 6.1 The changes over time in the proportion of trade events performed by Level 1 dairy herds where the selling herd was not in Level 1 at the time of the trade of cattle after the initiation of the surveillance program for *S.* Dublin in Denmark (Nielsen, 2009).

The national and regional changes in the percentage of BTM antibody positive dairy herds over time since the beginning of the testing programme in different regions of Denmark are illustrated in Figure 6.2. On the 27th June 2012, 293 (7.7%) of 3,803 active dairy herds were in Level 2 due to too high antibody levels, and another 11 (0.3%) were in Level 2 for other reasons, e.g. locks, contacts etc. (Source: www.kvaegvet.dk, accessed on 27th June 2012). The highest prevalence region, Himmerland, had 17.7% dairy herds in Level 2, 2R and 3 at that time.

The surveillance programme levels, 'Level 1' and 'Level 2', were evaluated in an International EpiLab project in Copenhagen in 2004-2005. The method used was a risk analysis model combining field data from previous field studies with a mathematical simulation model incorporating uncertainties and biological variations. It was estimated that 99% of Level 1 herds were truly non-infected and 80% (95CI: 68-84%) of Level 2 herds were truly infected at an underlying true prevalence of 15% (Warnick et al., 2006 **Paper XIV**). The results also indicated that when prevalence becomes lower, the PPV becomes lower which means that it may be necessary to find other ways or follow-up test-strategies to classify herds "most likely free from *S*. Dublin-infection" to avoid too many false positives. The false positive classifications are mainly due to a delay in the antibody decay after a recovery from an infection at herd level (Jordan et al., 2008 **Paper XI**), and cross-reactions in the used ELISA, even though other *Salmonella* serotypes that may give rise to cross-reactions are infrequently isolated in cattle herds in Denmark (Nielsen et al., 2011 **Paper IV**).

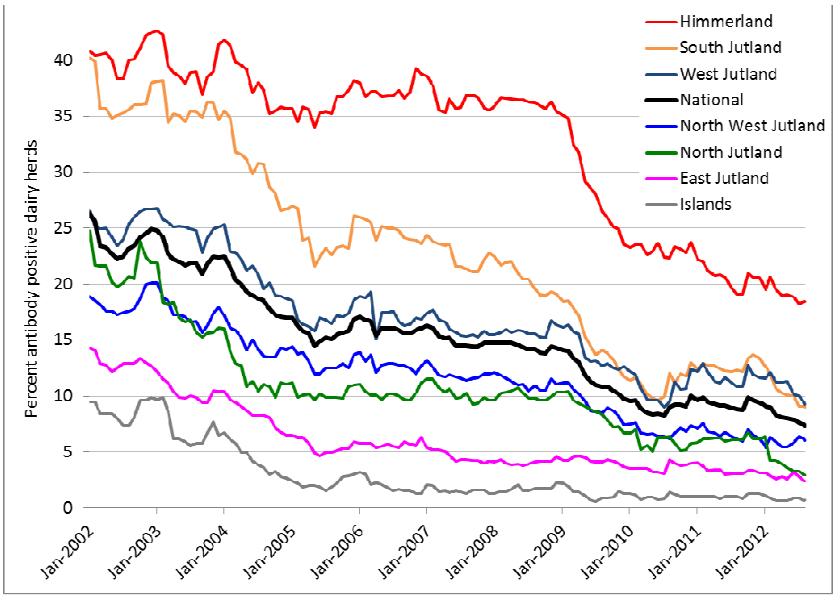

Figure 6.2 The percentage of dairy herds that have been antibody positive in the BTM sampling scheme since the beginning of the national surveillance program for *S.* Dublin in different regions of Denmark.

Herd classification of non-dairy herds

The large group of cattle herds (13,391 herds on the 20th June 2012) that do not produce milk in Denmark is a very mixed group that consists of small hobby herds, specialised veal production herds, heifer raising facilities of all sizes, special breeding herds for beef cattle, tradesmen and herds with a mixture of several production types. Both within-herd and herd level *S*. Dublin prevalence levels vary among these types of herds. Therefore, designing a testing strategy for non-dairy herds is a challenge. The research performed in this area concerns mainly specialised veal calf herds, because these pose the highest risk at slaughter due to the higher prevalence and the number of animals delivered to slaughter. In one Danish study, 2.3% of 1,703 tested slaughter calves from the 34 largest veal calf herds in Denmark in 2002 were found *S*. Dublin faecal culture positive at slaughter, and the association between the excretion of *Salmonella* bacteria at slaughter and an individual seropositivity at cut-off 10 ODC% in blood samples collected from the same animals was weak. It was discussed that this could be either due to delays in the time between an infection and the faecal excretion of bacteria and the serological response in the individual cattle (Anonymous, 2003). In total, 95% of all of the *Salmonella* serotypes found in that study were *S*. Dublin. In a later study of 71 randomly selected veal calf herds expected to deliver more than 100 veal calves to slaughter per year in 2007 and 2008, *S*. Dublin was cultured from faeces in 1.5% of all of the tested slaughter calves (n=1,296) (Nielsen et al., 2011 **Paper IV**). This sample of herds was more representative of the Danish veal producers than the study from 2002. Nielsen et al. (2011 **Paper V**) found that taking test accuracies into account and allowing for an unknown infection status in the analysis of the data, the most likely prevalence of infected veal producing herds was between 34% and 57%, and within the infected herds 21-49% were estimated to be infected at a slaughter age.

Accurate prevalence and test accuracy estimates are important when designing herd classification programmes for non-dairy herds, because the resulting HSe and HSp depend a lot on these estimates (Houe et al., 2004). Clearly, the most accurate classification could be obtained by testing all of the individual animals in the herd, but this is neither economically nor practically feasible. Thus, strategic samples have to be taken from each herd. In practice, the easiest place to sample animals is at slaughter immediately after the animal is killed, and this is what has mainly been used in the Danish surveillance programme for non-dairy herds. However, not all herds send enough animals to slaughter to get a useful classification, so blood samples can also be collected in the herds. Testing at the abattoir saves expenses on the sampling and provides a good opportunity to safely test unwilling or otherwise inaccessible beef cattle or large bull calves. Furthermore, automatic selection systems can be set up to point out animals for sampling, and samples collected at abattoirs can have the benefit of being properly labelled, e.g. with easy-to-read barcodes, and may be used for multi-diagnostics. In Denmark, many of the blood samples collected for BVDv and IBR-testing are also tested for *S*. Dublin antibodies.

In non-dairy herds it is not possible to get access to pooled samples that can easily be uniformly collected and tested using a diagnostic test with a high Se. Faecal culture methods, including the sampling of dung pits or pooled faecal samples, for a confirmation of suspected outbreaks are likely to have the same Se in non-dairy herds as in dairy herds. Knowing the Se and Sp of individual serum ELISA, it is possible to calculate the expected HSe and HSp given the number of tested animals. In the estimations illustrated below the ELISA Se was set to 70% and the Sp to 90% according to estimates reported by Nielsen et al. (2011 **Paper V**). Advanced methods for HSe and HSp estimations have been developed that take into account the uncertainty associated with sampling strategies in herds of different sizes and the uncertainties of test performance, e.g. (Jordan and McEwen, 1998; Greiner and Dekker, 2005). Online tools have been developed to aid with the calculations of a range of possible prevalence levels, test characteristics, herd sizes, sample sizes and threshold values for the number of positive tests leading to a positive herd diagnosis (e.g. Epitools: http://epitools.ausvet.com.au/, Accessed 28th December 2011). Figures 6.3 and 6.4 illustrate the effect of sample size (n) on HSe and HSp in non-dairy herds with a herd size of 10 and 100 at true within-herd prevalence levels of 5%, 10%, 20%, 30% and 40%. In Figure 6.3, a herd was considered positive if just one animal was test positive (>50 ODC%), in Figure 6.4 the herd was considered positive if at least two samples tested positive. The estimates were obtained using hypergeometrical distributions (HerdPlus) as described in Greiner and Dekker (2005). The estimates should be interpreted with a focus on the within-herd prevalence estimates (21-49%) found by Nielsen et al. (2011 **Paper V**), meaning that the red (p=20%), grey (p=30%), and black (p=40%) lines in the figures are the most relevant estimates of HSe for veal calf herds. HSe was very dependent on the within-herd prevalence, the number of samples collected and whether the criterion for the herd positivity is one or two test positive samples. The HSp decreased from around 85% to 50-60%, when the sample size was increased from 3 to 10 and if just one positive sample was the criterion for the herd to be considered positive (Figure 6.3). If the criterion for herd positivity was changed to two test positive samples the HSp was high, i.e. 90-100% depending on the herd size and the sample size (Figure 6.4). Figures for herd sizes 20 and higher (not shown) were very similar to the figure for 100 animals.

Obviously, a compromise is inevitable when selecting a sampling strategy in a monitoring or surveillance programme for *S*. Dublin based on blood sampling of non-dairy cattle. If the purpose

of sampling of the non-dairy herds is to protect the other herds from becoming infected upon contact or purchase, the HSe should be optimised in the classification system by using the one positive sample-criterion for a herd diagnosis.

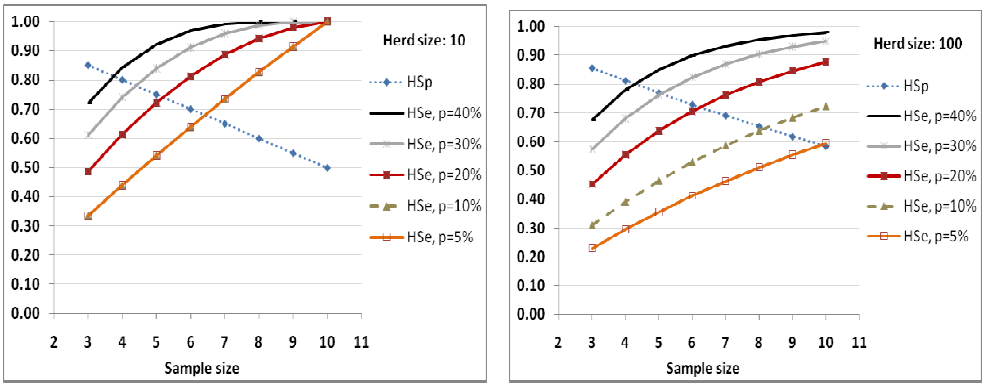

Figure 6.3 Estimated herd sensitivity (HSe) and herd specificity (HSp) for sample sizes ranging from 3 to 10 in non-dairy herds tested by *S*. Dublin serum ELISA at given underlying true prevalence levels (p) and herd sizes with 10 and 100 animals. In these estimations herds were considered positive if at least one sample had an ELISA reading above the cut-off value of 50 ODC%.

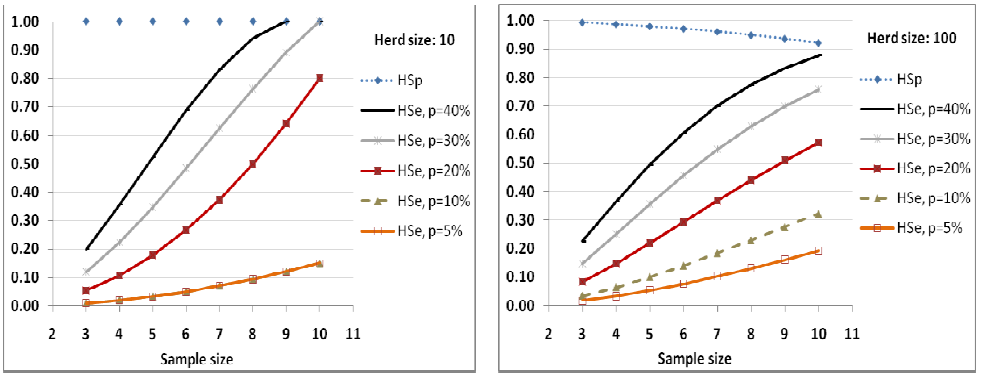

Figure 6.4 Estimated herd sensitivity (HSe) and herd specificity (HSp) for sample sizes ranging from 3 to 10 in non-dairy herds tested by *S*. Dublin serum ELISA at given underlying true prevalence levels (p) and herd sizes of 10 (left) and 100 (right). In these estimations herds were considered positive if at least two samples had ELISA readings above the cut-off value of 50 ODC%.

If the purpose of testing is to follow the development in a national prevalence over time or if false positive herd classifications have large consequences for the farmers, it might be worth considering the use of the criterion that would ensure a high HSp. This criterion, however, does not combine well with a reduced sample size, because HSe quickly becomes markedly lower when n is below 7.

Finally, it should be noted that test-strategies in non-dairy herds also depend on timing. If the samples are collected one at a time over an extended period, as in the Danish testing scheme, this may not be suitable for the detection of new infections, and herds classified as test positive may

have cleared the infection without it being registered in the system, unless the farmers requests extra samples themselves.

Surveillance of non-dairy herds

In the Danish surveillance programme for *S*. Dublin, non-dairy herds with 10 animals or more are classified according to the ELISA test results in the last eight blood samples usually collected at slaughter over time with no more than 180 day intervals (Anonymous, 2012b). For smaller herds the sample size is adjusted accordingly. If there are fewer than 4 animals in the herd, all of them must be sampled for a herd classification. If just 1 sample is test positive then the herd will be classified Level 2 until 8 new samples – either collected in the herd or at slaughter - are test negative. Because of the potentially long intervals between samples collected for surveillance, this system does not work well for the detection of new infections. Detection of new infections therefore relies on the passive surveillance component that dictates that it is the duty of the veterinarian to submit samples for bacteriological culture to seek a diagnosis, if suspicion of salmonellosis arises in a herd (Anonymous, 2012b). Confirmation of a new infection should preferably be done soon after any suspicion arises. If faecal culture is used for a confirmation, clinically ill and non-treated cattle should be sampled as soon as the clinical signs are noticed to optimise sensitivity. If serology is used, the testing should be performed at least two weeks after the clinical signs have started, and the group that is suspected of an infection should be sampled unless they are below 12 weeks of age. If that is the case, it might be worth waiting to use serology tests until that group of animals have reached the right age. A sample size of 10 animals is a sensitive herd testing strategy, but if only one animal has a high ELISA response and all of others have low ELISA results, it might be either because of a very low prevalence (unlikely in outbreak herds) or a false positive reaction that warrants some follow-up investigations in the herd.

From the beginning of the surveillance programme in 2002, the herd classification method for non-dairy herds was based on three blood samples from each herd. However, in 2006 it was changed so that non-dairy herds are currently classified as follows:
Level 1b ('most likely to be free of *S*. Dublin based on low antibody levels in blood samples') is obtained if the last eight blood samples from the animals aged 3 months to 5 years are < 50 ODC%, and it is more than 3 weeks ago that the herd was in Level 2 and more than 6 months ago that the herd was in Level 3. If the herd doesn't live up to these criteria, then the herd is placed in Level 2. Furthermore, a new Level 2R was introduced in 2010, which places the herd under movement restrictions until it can subsequently be tested negative using blood samples from the 10 youngest calves above 3 months of age (Anonymous, 2012b). Diagnosed clinical salmonellosis leads to Level 3 as it does in the case of dairy herds.

Herds that do not have enough samples collected to be classified are placed into an "unknown" level (Anonymous, 2006a). Antibody measurements do not outdate unless the herd changes to Level 2, which means that over time there will be fewer and fewer herds in the unknown level. On 27th June 2012, there were 4.0% of the antibody positive non-dairy herds out of a total of 13,358 herds, and another 21.5% that could not be declared as Level 1 either due to missing samples or contact with Level 2-herds. As shown in Table 3.2 the proportion of herds in the unknown status due to missing samples is highly correlated with the average number of slaughtered cattle per year. The same locking system counts for non-dairy as for dairy herds. About half of the approximately 535 herds in Level 2 are veal calf herds or heifer raising facilities, both are

characterised by continuously receiving a large proportion of their calves and young stock from dairy herds. An unspecified part of the “Other herds” are dairy herds that recently went out of business.

Animal level test interpretation

In a passive surveillance of *S*. Dublin, the animal level diagnosis is relevant for a confirmation of clinical suspicions. Bacteriological culture methods have been the traditional method of choice for this purpose despite the low Se. One reason for this preference is that in clinical outbreak situations, it is often useful to know the serotype to trace the potential source of the infection. Another reason is that the Sp of serology is imperfect, which may lead to a lack of confidence in positive test results, in addition serology cannot differentiate between serotypes with certainty (Konrad et al., 1994). Moreover, in clinical outbreaks the Se of faecal culture can be expected to be much higher than in endemically or subclinically infected herds due to higher concentrations of bacteria being shed from acutely infected or clinically affected susceptible animals (Steinbach et al., 1996; Christensen, 2005). The overall PPV for the faecal culture test of *S*. Dublin can be assumed to be close to 100%. This means that if the test is positive, we can be certain that the animal was shedding bacteria in faeces at the time of sampling. This is independent of the underlying prevalence. The estimated NPV of individual faecal culture at different prevalence levels of infection in the herd were estimated by Nielsen et al. (2004b). The estimates varied from 0% to 13% and were highest at the lowest underlying prevalence. In other words, if a faecal culture test is negative, it does not tell us much about the true infection status of the tested animal. Animals tested with bacteriological culture methods should not have been treated with antibiotics prior to sampling. This would lower the Se and the NPV even further.

In control programmes for *S*. Dublin, the purpose of testing individual animals can be either to detect potentially infectious individual animals for risk management (e.g. to let high risk cows calve in single pens that are cleaned after each use), culling or to evaluate the effect of control measures (e.g. testing of a group of calves that the farmer believes has not been exposed to *Salmonella* after he has changed some management practices 6 months prior to this event). For purposes related to the evaluation of progress, serology has proven superior to bacteriological culture both regarding predictive value and costs. The individual animal serum and milk ELISAs have NPVs that are 2-10 times higher than faecal culture depending on the underlying prevalence, whereas the positive predictive value of ELISA as an indicator of an *S*. Dublin infection in an animal varies tremendously with the underlying prevalence when interpreted on the basis of a single measurement (Nielsen et al., 2004b).

In practice it does not make sense to use single ELISA results to determine the current infection state of an animal. The dynamics of antibodies upon a new infection and repeated infections need to be considered (Robertsson, 1984). In the field study described in Nielsen et al. (2007 **Paper III**), both calves and their dams were sampled once or twice per week. Figure 6.5 illustrates an example of the antibody responses measured in a cow that became infected 4 weeks after calving. Sampling was started at the time of calving. Faecal shedding was detected for approximately one week. Seroconversion was measurable two weeks after the cow started shedding bacteria. The practical use of the above information will be discussed in Chapter 7 concerning the control of *S*. Dublin in cattle herds.

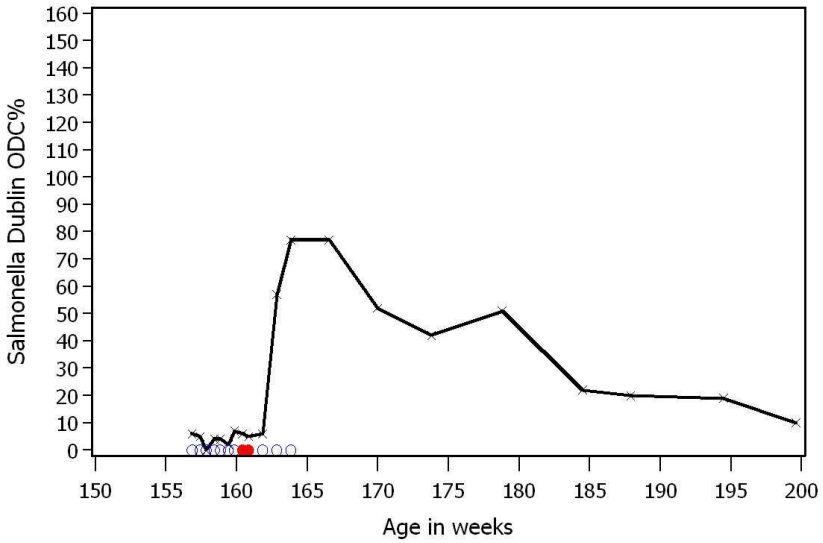

Figure 6.5 A typical course of faecal excretion (red dots) and the antibody responses measured by individual milk ELISA in a dairy cow that became infected 4 weeks after calving, then it excreted bacteria in faeces for approximately 1 week, it seroconverted after approximately 2 weeks and appeared to clear the infection allowing its antibodies to return back to low levels (below 25 ODC%) approximately 5 months after the infection.

House et al. (1993) suggested to cull repeatedly seropositive animals due to a suspicion of them being *S*. Dublin carriers with a higher risk of shedding. Other studies have suggested that a testing strategy to differentiate between persistently infected carriers and transiently infected cattle could be to test for antibodies twice or three times at 120-days intervals (Spier et al., 1990; Smith et al., 1992). However, the study material used in those studies did not include representative samples of animals, so the results could be biased. Therefore, the probability of *S*. Dublin positive faecal cultures was investigated in all animals ≥180 days old with different temporal antibody profiles in 14 endemically infected Danish dairy herds that were sampled four or five times at 3 month intervals between 2000 and 2002 (Nielsen, 2013 **Paper XII**). Overall, there was very few positive faecal cultures 46 (0.7%) of the 6,614 observations. This complicated the further analyses somewhat. The proportion of observations that represented animals with persistently high (≥80 ODC%) antibody levels was low (on average 2.8% across all herds). The same was the case for animals with recent increases in antibody levels from <25 ODC% to (≥50 ODC%). There were 3.2% of those. A hierarchical multivariable logistic regression analysis was performed including four temporal antibody profiles and age of the animals, and the results are illustrated in Figure 6.6. Less than 30% of the cattle with such temporal antibody profiles posed a risk of spreading the infection, and the highest risk (~5%) was found in cattle < 1-2 year old with a recently high antibody measurement. This complicates test-and-cull strategies as a tool in control and eradication programmes. In adult cattle > 3 years old, the probability of a positive faecal culture was found to be low (<2%) regardless of the measured antibody levels or temporal antibody profiles.

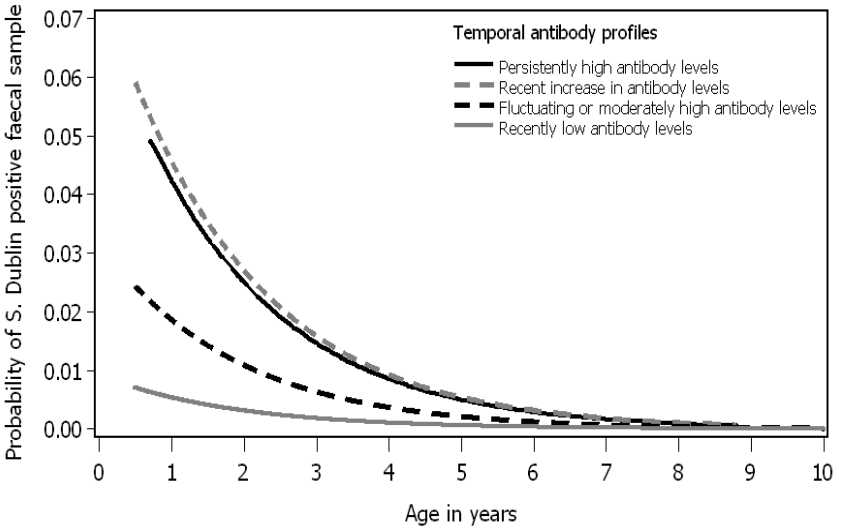

Figure 6.6 Model predicted probability of *S.* Dublin positive faecal culture in four different temporal antibody profiles vs. age of the animals on the date of the most recent sampling (Nielsen, 2013 **Paper XII**).

Veling (2004) suggested to use faecal cultures for the detection of actively shedding carriers in seropositive cattle that are in persistently infected herds. However, the very low sensitivity of this method may lead to unsatisfying results. I suggest that faecal culture can be used to test individual cattle in a group of animals that show signs that there might be an actively excreting carrier in the group. Figure 6.7 illustrates ELISA measurements in all young stock in a dairy herd 1 year into the control programme planned for that herd in the study by Nielsen and Nielsen (Nielsen and Nielsen, 2012 **Paper XV**).

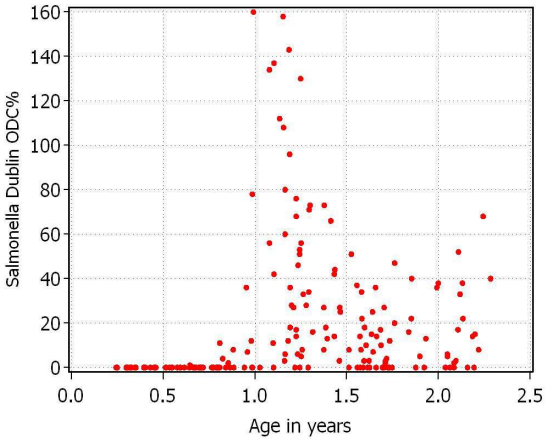

Figure 6.7 Example of antibody ELISA response (ODC%) vs. age in years at sampling used to evaluate progress in control of *S.* Dublin in young stock and lactating cows. In this particular example there are no signs of infections having occurred in cattle below one year of age suggesting that control actions have had a good effect in this part of the herd (Nielsen and Nielsen, 2012 **Paper XV**).

In Figure 6.7 there is clear evidence that the spread of *S*. Dublin among calves and young stock up to 1 year old has ceased, because none of the animals have measureable antibody responses against the infection. However, in the heifers from 12 to 15 months which were housed together the very high antibody responses in several of the animals suggest that spread of the infection is on-going and it is likely that there is a continuous source of bacteria, such as an active carrier in the group that might be detectable by use of faecal culture.

Several of the heifers with persistently high antibody profiles from this herd and other herds were later purchased for an experimental study (Lomborg et al., 2007 **Paper XIII**). In that study, only 30% of the suspected carriers were found to have *S*. Dublin bacteria in the internal organs, and they did not shed the bacteria in faeces at any point in time during the study period, despite being immunosuppressed experimentally. House et al. (1993) found 3% of faecal samples and 2.5% of milk samples from eight suspect carrier cows *S*. Dublin culture positive, when they were sampled five times per week for 6 months. In contrast, five suspected carrier calves (average age 7 months) from the same dairy herd were found *S*. Dublin culture positive in 17% of all faecal samples under the same sampling scheme. In conclusion, it appears to be necessary to develop herd-specific approaches to prevent the spread of bacteria to control of *S*. Dublin in cattle herds rather than relying on culling suspected carriers.

Chapter 7
Control and eradication of *S*. Dublin

This chapter provides an introduction to the Danish approach to control *S*. Dublin in the cattle population. All of the papers in this thesis have provided new knowledge and scientific evidence to support decisions on the chosen approaches and methods to go by nationally, or provided recommendations to farmers who want to control the infection in their herds. Paper XI is specifically concerned with control at a national level, and papers XV and XVI focused on investigating and demonstrating an approach to control *S*. Dublin in infected herds.

National control of *Salmonella* in general

The ultimate purpose is that Denmark may eventually become able to apply for a special "*Salmonella* free" status in the EU, and if this is granted it will be able to apply restrictions on the import of *Salmonella* contaminated meat from other countries (Anonymous, 2006). This involves all of the sectors of production animals. However, the different sectors have been approaching the control of all serotypes of *Salmonella* in different ways (Wegener, 2010). The first target of the overall plan was reached in April 2012, when the EU granted Denmark 'negligible risk of *Salmonella* in eggs for consumption', which essentially allows the Danish authorities to require the same low risk in imported eggs. The cattle industry decided to focus on *S*. Dublin, because it is the most prevalent infection in cattle herds.

Control of *S.* Dublin at national level

In 2006 an agreement was made between the Danish Cattle Federation and the Danish Veterinary and Food Administration that a control campaign was to be started from 2007 with the aim of eradicating *S*. Dublin from the Danish cattle population before the end of 2014. According to Yekutiel (1980) eradication as a concept that can be formulated as: 'The purposeful reduction of specific disease prevalence to the point of continued absence of transmission within a specified area by means of a time-limited campaign'. For the *S*. Dublin eradication campaign in Denmark this was further specified to imply that the prevalence has to be close to 0% of the infected cattle herds, there should be no more than five newly infected herds per year and the infection in these herds must be effectively controlled, so that it does not spread to other herds. Having the surveillance programme in place and farmers who are used to the surveillance programme levels has provided some useful tools in the control programme as described below.

Paper XI describes work providing decision support for the design of the control programme for *S*. Dublin in the dairy cattle population by Jordan et al. (Jordan et al., 2008 **Paper XI**). The results of the study were specifically used by the working group of the *S*. Dublin surveillance and control programme led by the Danish Veterinary and Food Administration in their action plan report to the Minister of Food, Agriculture & Fisheries in 2009 (Anonymous, 2009a).

The scenarios investigated by the simulation model in that study are summarised in Table 7.1 together with the estimated prevalence of *S*. Dublin after 10 years resulting from each control scenario. Scenario 1 (base scenario) was the scenario assumed to resemble the control elements in the surveillance programme in 2006. Figure 7.1 shows the simulated change in apparent and true prevalence at a national level over a 10 year period in one of the simulated iterations starting from

that time. Figure 7.2 shows the simulated distribution of true and apparent prevalence levels of *S*. Dublin infected dairy herds after 10 years following the base scenario.

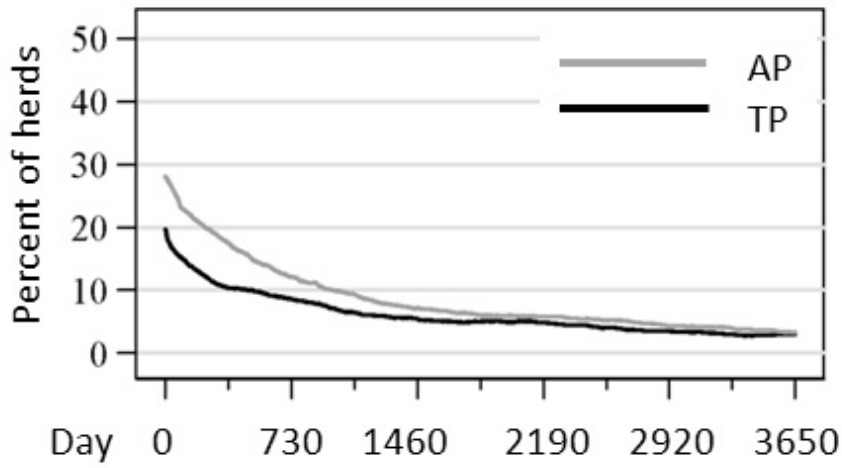

Figure 7.1 The predicted national true prevalence (TP) of cattle herds infected with *S*. Dublin and the prevalence of herds classified as Level 2 (AP) under Scenario 1 (base scenario) from a single iteration of 3650 days (10 years) (Jordan et al., 2008 **Paper XI**).

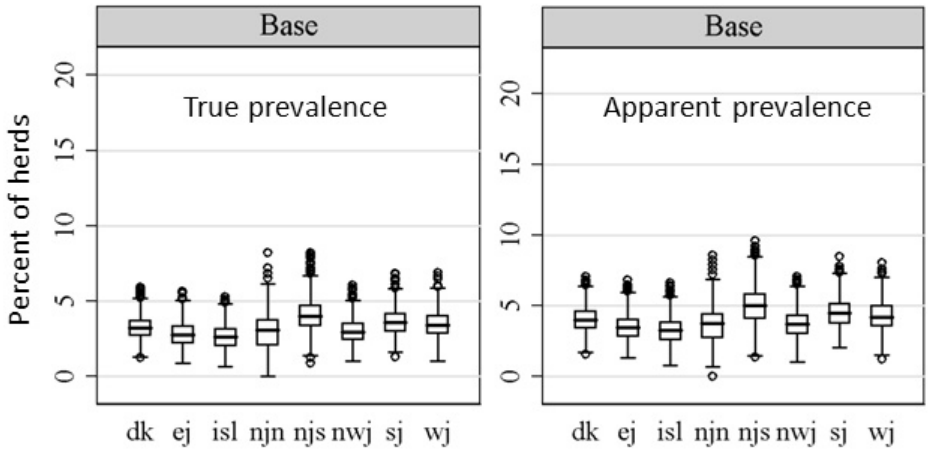

Figure 7.2 The predicted true prevalence of *S*. Dublin infected cattle herds and the apparent prevalence (AP) of infected herds after 10 years in Denmark (dk) and seven regions (East Jutland (ej), the Islands (isl), the northern part of North Jutland (njn), the southern part of north Jutland (often known as Himmerland, njs), North West Jutland (nwj), Zealand (sj) and West Jutland (wj) under Scenario 1 (the base scenario) based on 1000 iterations (Jordan et al., 2008 **Paper XI**).

Assuming that June 2012 was 6½ years into the base scenario simulations, the iteration displayed in Figure 7.1 actually resembles the current situation very well (7.7% antibody positive dairy herds by 27th June 2012). The displayed iteration suggests that the prevalence would reduce slowly after

this and end at around 3% after 10 years, which would not be sufficient to reach the goal of the control programme. It was concluded that additional control measures were needed. The results suggested that an enhanced internal and external biosecurity in both infected and non-infected herds were the preferable methods to reach the goal of an eradication of *S*. Dublin from the cattle population by 2014, preferably combined with an early warning system to detect newly infected herds faster than today (Scenario 4). The results also suggested that restrictions on cattle movements between regions would have to be combined with other control elements such as an improved internal biosecurity in infected herds to reduce the prevalence sufficiently (Jordan et al., 2008 **Paper XI**).

However, one important issue that the model did not take into account was the structural changes in the cattle industry. The number of dairy herds decreased from more than 7,000 in 2002 to 3,800 in 2012, and the number continues to decrease. The number of cows has not decreased equivalently, so herd size has rapidly increased with new and larger farms being built and more multi-site herds appearing. Hence, it was unavoidable to move cattle between herds to a greater extent than simulated in Scenario 3 (i.e. improved external biosecurity), which was otherwise one of the most efficient strategies according to the model. Furthermore, the environmental exposure probability (EEP) used in the model was uncertain, and may in fact differ markedly between regions with different prevalence levels (Nielsen et al., 2007 **Paper VI**; Ersbøll and Nielsen, 2011; Nielsen and Dohoo, 2012 **Paper VII**; Nielsen and Dohoo, 2013 **Paper VIII**).

Economic considerations were not taken into account in the model by Jordan et al. (2008 **Paper XI**). In a Dutch simulation modelling study, different control strategies were ranked according to the effect on herd level prevalence after 10 years, costs and cost-effectiveness ratios. They found that a compulsory control strategy which included the culling of chronically infected animals from infected herds, which was assumed to shorten the duration of the infection in the herds, alongside prohibiting the purchase of potentially infected animals would reduce the prevalence of *Salmonella* positive herds from the starting point at 8.3% to 0.8% over 10 years in the most cost-effective way. Adding hygienic measures and a ban on the movement of animal manure would reduce prevalence slightly more, but at substantially higher costs (Bergevoet et al., 2009).

In conclusion, an effective control programme needed to include elements to improve internal biosecurity in infected herds aiming at reducing the duration of infection in infected herds. How this can be obtained is discussed below. External biosecurity is also essential, including a reduced or a complete stop to the movement of animals from infected herds to other herds, markets and pastures, alongside measures to reduce the effect of some of the diffuse risk factors in the Danish cattle population, such as a controlled spread of manure from infected herds on fields that cattle could gain access to (Taylor and Burrows, 1971; Findlay, 1972; Allerberger et al., 2003).

Table 7.1 The control scenarios and results from a simulation model of *S.* Dublin in the Danish dairy cattle industry (Jordan et al., 2008 **Paper XI**)

Scenario # and name	Short description of scenario	Predicted median national herd prevalence after 10 years
1 Base scenario	Approximates the current management of *S.* Dublin according to the national surveillance program. EEP[a]=10^{-5} Herds were allowed to acquire replacement animals from any other herd regardless of region by only taking into account their simulated purchase policy. BTM-ELISA testing was performed at the usual 90-day interval.	3.25%
2 Regional restriction of animal movement	Herds seeking replacement animals could only acquire them from the herd's home region. This prevents high-prevalence regions from 'exporting' infection thereby protecting the low-prevalence regions from external sources of *S.* Dublin infection. EEP=10^{-5} BTM ELISA testing was performed at the usual 90-day interval.	3.38%
3 Enhanced external biosecurity	Limited all of the herds to no more than 12 purchase events per year and the number of animals acquired at any one purchase to 12. The proportion of the herds with an 'indiscriminate' purchasing policy and the proportion of the herds with a 'conservative' purchasing policy were halved with the remaining proportion assigned a purchasing policy of 'closed'. EEP=10^{-5} and BTM ELISA testing was performed at the usual 90-day interval.	0.1%
4 More frequent herd testing	Herds were tested more frequently by reducing the interval between BTM ELISA tests to 30 days. EEP=10^{-5} and original purchasing policies.	1.55%
5 Enhanced control at herd level	Reduced the duration of time that individual herds spent in the true-positive period to half the mean of the exponential distribution used to model the true-positive period in the base scenario 1. (In scenario 5 a mean of 338 days was used). EEP=10^{-5} and BTM ELISA testing was performed at the usual 90-day interval.	0.18%
6 Combination of scenario 2, 3, 4 and 5	Combined all of the features of scenarios 2, 3, 4 and 5 to provide some indication of the maximum possible reduction in prevalence that might occur with this composite approach.	0%

[a]EEP The environmental exposure probability is a variable in the model that encompasses all of the exposures to infection other than those caused by contact with an infected animal.

The Danish *S*. Dublin control and eradication programme

Here follows a presentation of the Danish eradication plan for S. Dublin. Some information about this programme is needed to understand the background and discussion of the results from some of the next studies that will be presented. The programme was organised into three phases.

Phase 1, a voluntary control in infected cattle herds from the beginning of 2007 to the end of 2009. In this period the surveillance program and the regulations regarding *S*. Dublin were not supposed to be changed. The Danish Cattle Federation ran a campaign to encourage farmers with infected herds to start controlling the infection, and farmers with uninfected herds to protect their herds carefully. The campaign consisted of an information strategy to reach as many farmers and advisors as possible, but also included several research and demonstration projects in the field. All of the dairy herds that were in Level 2 and Level 3 in that period were offered a place in a network group of 6-8 farmers that met several times together with a cattle consultant or veterinarian acting as a facilitator. This system is also referred to as 'stable schools' (Vaarst et al., 2007). Almost half of the farmers that received the offer used the opportunity. Much of what needs to be done in a herd to eradicate *S*. Dublin concerns daily management routines, so risk assessment tools have been developed to aid the farmers in deciding the most optimal control strategy in his/her herd, and test interpretation tools have also been made available as described in more detail below.

Phase 2, an intensified control campaign running from 2010 to 2012. Price differentiations on milk and beef meat were originally planned to improve the incentives to eradicate *S*. Dublin from cattle herds. However, due to the financial crisis in agriculture in 2009-2010 the plans were changed. Instead a new surveillance level was introduced, Level 2R, and the legislation was adjusted accordingly. The idea was to introduce stricter restrictions on Level 2R movements, so that these herds could only deliver live cattle to slaughter and were not allowed to move them between other herds. Dairy herds were placed in Level 2R, if they had >40 ODC% in one of the last two BTM ELISA measurements, and non-dairy herds if they had at least one blood sample with >50 ODC%. It became evident that this change would make it very difficult for many Level 2R dairy herds to get rid of their bull calves to slaughter calf producers, which would lead to an overcrowding of animals in many infected dairy herds. So, two doors were opened in the legislation: (1) calves could be sold from Level 2R directly to specialised veal calf producers, who signed consent forms to receive the calves from Level 2R herds; and (2) the calves could be sold for export.

Phase 3 was planned to be introduced in 2013 and 2014. The Danish Veterinary and Food Administration may use new executive orders or changes to the current legislation about *Salmonella* in cattle to prevent the spread of infection from *S.* Dublin-infected herds, for instance by closing down herds that are entirely for the trade of living animals, compulsory action plans with advisors and follow-up testing to evaluate the progress in the control efforts. However, by the beginning of August 2012 work was still on-going to decide the specific actions in phase 3.

As described in detail in Chapter 4, the analysis of the hazard of recovery from *S*. Dublin infection over time in the surveillance and control programmes were investigated by Nielsen and Dohoo (2013 **Paper VIII**). An important conclusion of that study relevant for design of control programmes was that recovery was markedly more likely (and the duration of an infection markedly shorter) for up to 2 years after the new central initiatives were started up which supported the decision to have a voluntary phase in the beginning of the control campaign in which control of *S*. Dublin was strongly encouraged. The first period with increasing and high

recovery rates was in 2003-2005 after the national surveillance programme was initiated, and the second period was from late 2007 and throughout 2009 when the intensified control campaign was running with projects and information targeted towards the infected herds. There are, however, signs that the positive effect of the control campaign waned from 2010 and up to beginning of 2012.

By 27th June 2012, there were 59 specialised veal calf producers in Denmark that had received calves from Level 2R. In 2011, 40,554 cattle were exported out of Denmark. Out of these 25,157 were calves <6 months old that were exported to the Netherlands for veal production. Many of these came from Level 2 and Level 2R, because the price of the calves from these herds was markedly lower than calves from Level 1 in Denmark. Calves from the few herds in Level 3 cannot be exported or moved to other herds. However, there have been very few herds in Level 3 since 2007, and information from field veterinarians tells us that it is often due to the fear of movement - and other troublesome - restrictions that farmers do not allow samples to be collected and submitted upon suspicion of salmonellosis despite the fact that it is dictated in the legislation that they must allow this. This is a dilemma for the veterinarians and farmers, but it is likely that these "open doors" in the control programme lead to a lack of incentives to control *S*. Dublin in too many of the remaining infected herds. The conclusion must be that new incentives are needed to reach the goal of the programme: to eradicate *S*. Dublin from the Danish cattle population by the end of 2014. Even though cost-benefit calculations have not been performed in Denmark for the national control scenarios, the size of the on-going losses in infected herds leaves room for quite substantial costs for control measures to reach break-even. A frequently encountered argument is that the owners of infected herds need to carry the costs for control procedures themselves, because it is in their own interest. However, if a herd owner does not understand the benefit or is not capable of managing their herd through a successful control programme, his/her herd can continue to spread the infection to other 'innocent' farms with potentially significant losses as the consequence (Nielsen et al., 2013 **Paper X**).

Since the aim is to eradicate *S*. Dublin, it is also worth looking at approaches from countries with a very low prevalence. In Sweden, whenever any serotype of *Salmonella* is isolated, restrictions are immediately placed on animal and manure movements from the infected farm(s). Furthermore, a prompt investigation and trace-back of the infection is performed. An eradication plan is formed by an official veterinarian and approved by the Swedish Board of Agriculture. The herd is not declared free from infection until all of the animals in the herd are negative in two consecutive faecal samplings of every animal one month apart, and adequate cleaning and disinfection have been completed (Lewerin et al., 2011). Bacteriological culture is the detection method of choice for most samples from animals, because it has a high specificity which is important for the Swedish situation. However, as mentioned in Chapter 6, this is a very expensive sampling strategy, so other options should be sought and the testing procedures described in this thesis may be relevant.

Control of *S*. Dublin within dairy herds

A study from the Netherlands suggested that approximately half of the dairy herds that experienced an outbreak of *S*. Dublin became persistently infected (i.e. still had signs of new infections one year after the outbreak), and the probability that the infection became persistent in the herd depended on how well transmission was limited early in the outbreak. Furthermore, it was found that persistence could not be avoided solely by culling active carriers detected by faecal sample cultures, but that management procedures directed against a spread of the infection were

required (Veling, 2004). Therefore, the first advice must be to try to limit the spread of infection, if an outbreak occurs. Isolation of clinically ill animals is often suggested as a way to reduce the exposure of other animals, and this makes sense, because they often excrete high numbers of bacteria. However, other animals in the herd are also going through the full infection cycle and shed bacteria even though they do not show any clinical signs, so it is not enough to target control actions towards the clinically ill animals (Nielsen et al., 2007 **Paper III**).

Management procedures that have been shown to prevent the infection of calves in dairy herds include practices that prevent any contact between infected and susceptible cattle, and hygiene which reduces the contamination of the environment, e.g. good and consistent calving management including good hygiene where cows are calving, such as having few cows in the calving area simultaneously and not using the calving pen for sick cows. Furthermore, good colostrum handling practices and separation of pre-weaned calves that prevents a spread of manure between neighbouring calves have been shown to be important control measures (Jensen et al., 2004; Nielsen et al., 2012 **Paper IX**; Nielsen et al., 2012b). Infection is possible via aerosols (Wathes et al., 1988). Therefore high pressure cleaning of buildings and pens is a high risk procedure, if live animals are present. The buildings should be allowed to dry well before new susceptible animals enter the area after such cleaning procedures (McLaren and Wray, 1991; Wray et al., 1991; Carrique-Mas et al., 2008).

Transmission from adult carriers to calves has been described (Richardson, 1973). This is important around the time of calving when contact between the carrier and the calf is most intense. In this situation the carriers are subject to stress, which may lead to a reactivation of a latent infection or an increased excretion of the bacteria from carriers (Spier et al., 1991; Kehrli et al., 1999). Carriers do not only pose a risk to their own calves in the calving area. If no measures are taken to avoid cross contamination to the next calving cows and their calves, these may also become infected, and calves are highly susceptible just after birth (Fisher et al., 1976). Excretion from carriers can contaminate the environment both indoors in barns and on pasture. However, even without the carriers infection can remain persistent in a herd as long as there is enough entry of susceptible animals and/or sufficient environmental contamination from acutely infected animals (Nielsen et al., 2007 **Paper III**; Nielsen et al., 2012 **Paper IX**).

It can be difficult to decide, which control procedures are needed for individual herds, and to ensure the daily and long-lasting control efforts required for success. Therefore, a structured approach based on a step-wise procedure to control *S*. Dublin in dairy herds focused on the support of farmers and their advisors in the planning of successful control programmes was developed and assessed in collaboration with 10 Danish dairy herds from 2003 to 2006 (Nielsen and Nielsen, 2012 **Paper XV**).

The five steps in the structured approach were:
1) risk scoring to determine transmission routes within the herd and into the herd;
2) determining a plan of action;
3) performing management changes to close important routes of infection;
4) interpreting repeated test results of individual animals to detect high-risk animals for special hygienic management or culling; and
5) diagnostic testing of different age groups and BTM to evaluate the progress of control over time.

The risk scoring forms (Step 1) are provided as online supplementary material to the paper by Nielsen and Nielsen (2012 **Paper XV**). The forms take the user(s) through the relevant barn sections and encourage a systematic evaluation of the hygiene and management practices with the herd. It is meant as a didactic tool for the users. The risk scores are subjective and meant to stimulate a discussion and exchange of information about the current management to provide a common understanding among the users (e.g. herd owner, manager, employees and advisors). After having used the risk scoring system to point out the most important action points, a control action plan can be established (Step 2) and carried out (Step 3). In the paper it was suggested that management procedures may be supplemented by test-and-manage procedures based on the repeated sampling of individual cattle in the herd (Step 4). Empirical experience and simulation modelling results suggest that it is not worth starting Step 4 until transmission has evidently been stopped among young stock (Bergevoet et al., 2009; Nielsen and Nielsen, 2012 **Paper XV**). Furthermore, farmers are unlikely to cull suspected carriers based on repeated antibody measurements as long as there are many heifers and cows with repeatedly high ELISA measurements in the herd (Nielsen and Dohoo, 2011 **Paper XVI**). This may also be a bad idea according to the results presented by Nielsen (Nielsen, 2012 **Paper XII**), because the risk posed by animals with persistently high antibody levels is only slightly higher than the rest of the seropositive animals in the herd. Follow-up is required on a regular basis at least until there are clear signs that the spread of an *S*. Dublin infection has been stopped in the herd. The only way to evaluate this is to test the relevant age groups of cattle in the herd (Step 5). BTM ELISA testing will only show, whether the antibody levels among the lactating cows is going down as an indication that bacteria are no longer spreading in the cow barn (Nielsen and Ersbøll, 2005; Nielsen and Nielsen, 2012 **Paper XV**). It is the most uncertain way to evaluate progress and is often delayed compared to the effect control might have on calves and young stock. The best indicator group to test early in a control programme is calves from 3 to 6 months old. It would be useful to be able to test younger calves, but both serology and faecal culture have too poor a sensitivity to use for this purpose. However, testing the 10 youngest calves above 3 months of age would lead to a HSe of at least 85% at cut-off 50 ODC%. HSp is better than shown in Figure 6.5, because individual animal Sp is much better in young calves than in older young stock and adult animals (Nielsen and Ersbøll, 2004). If the tested calves in this age group do not all have very low antibody levels in serum it is a sign that transmission is still on-going either in the calving area or between young calves, and this transmission route must be blocked if *S*. Dublin is to be controlled.

The culling of potential carriers may be beneficial to avoid re-infection of the herd once management actions have been implemented successfully to stop a spread of infection and when prevalence is low. However, due to the intermittent shedding patterns of most carrier animals it is not possible to accurately differentiate active carriers from non-shedding animals with a persistently high serology. In some herds there will be many potential carriers on the culling list and it might not be profitable to cull them all (Bergevoet et al., 2009; Weber et al., 2009; Nielsen and Dohoo, 2011 **Paper XVI**). Testing too early after an outbreak might result in too many test positive animals for culling. Testing at a later stage will increase the infectious period of the herd. Sensitivity analysis indicated that the optimal time to start testing was 11–13 months after the initial outbreak (Bergevoet et al., 2009). It is, however, recommended to investigate whether it is necessary and/or profitable to cull animals in the herds during a control programme using simulation modelling of specific herd scenarios where herd size, management practices, calf mortality, reproduction management, culling strategies etc. can be taken into account. It may very

well be that recommendations that are good for one herd will not work or be too expensive in another herd. Therefore, simulation of control scenarios for dairy herds including test-strategies are planned using the Dublin-Simherd model previously described (Nielsen et al., 2012 **Paper IX**; Nielsen et al., 2013 **Paper X**).

Control of *S.* Dublin in non-dairy herds

Control of *S*. Dublin in non-dairy herds can be performed according to the same principles as suggested for the dairy herds above. However, some types of non-dairy herds have challenges that are less common in dairy herds. Heifer raising facilities and slaughter calf producers have to move calves or young stock into their herd, often mixed from several dairy herds. Their production relies on these animals, and therefore an important part of the control plan is to communicate with the suppliers and demand that these herds deliver *Salmonella* free animals. This is happening to an increasing extent in Denmark, which is also one of the reasons why it is becoming increasingly difficult for Level 2 herds to sell their bull calves in Denmark. In many beef herds a common challenge is the close, long-term contact between dam and sucklers.

Vaccination

The efforts to develop vaccines against *S*. Dublin have had varying and mostly limited success. Calf mortality was significantly reduced in calves given two doses of a modified live, genetically altered *S*. Dublin-vaccine subcutaneously at 24 and 31 days of age, respectively, compared to a non-vaccinated control group. The study was performed as a double blind field study in one large dairy herd with a long history of *S*. Dublin infection and associated clinical problems (Selim et al., 1995). According to the authors, the *Salmonella* immunogen employed in that study was a genetically altered modified live '*aro- S.* Dublin', which had a gene deletion in gene *aro* A, making it unable to multiply appreciably in mammalian tissues or to survive in the environment. The advantage of employing live vaccines is that they are capable of inducing cell-mediated immunity, which appears to be important in eliciting a satisfactory immune response to *Salmonella*. However, this vaccine would be inactivated by antibiotic treatments, which in the study herd meant that the calves could not be vaccinated until 24 days. Most of the calves that died in that herd, died before they were 2 weeks old, mainly due to *E. coli* septicaemia, and were therefore never immunised against *S*. Dublin (Selim et al., 1995). Faecal shedding was not evaluated in that study, neither for wild-type *S*. Dublin nor for the modified vaccine strain. There were 250 calves in each of the study groups. However, it is uncertain how many of these were exposed to *S*. Dublin.

In a study of an oral *S*. Dublin-vaccine with the same genetically altered aromatic-dependent (*aro*-) *S.* Dublin strain, doses of 1.7×10^{10} were given twice at the age of two and four weeks followed by an infection with a virulent *S*. Dublin-strain (T2340). Protection was not evident and most of the calves (vaccinated or not) died upon the challenge (Smith et al., 1993). Similar results were found in a field study of neonatal calves (Habing et al., 2011).

In another study, it was shown that giving a vaccine with an avirulent live *Salmonella* choleraesuis ('strain 54') subcutaneously or intranasally to protect calves against salmonellosis caused by *S.* Dublin led to significantly fewer clinical signs, less shedding and a faster recovery of bacteria from the organs, but did not entirely prevent the disease and the shedding of the inoculate strain (Fox et al., 1997).

Segall and Lindberg (1993) found that it was possible to improve the immunity of calves around the age of 5 to 7 weeks using an oral live vaccine *S*. Dublin-strain as a vaccine. The vaccine was given as three weekly increasing dosages of a so-called *S*. Dublin (O9, 12) hybrid strain SL7103 which only gave rise to mild, transient increases in temperature. Upon infection with high doses of a known virulent strain of *S*. Dublin (SVA47) calves exhibited only transient fever and a mild mucoid diarrhea, which is a much milder course of infection than is seen in naïve calves. It did not, however, stop the infection from spreading to enterocytes of the jejunum and ileum, follicle-associated epithelium over the Peyer's patches and glandular tissues of the duodenal and tonsillar areas in the lungs.

An alternative approach was tried by Staak et al. (1989) who infused heat-inactivated *S*. Dublin-bacteria into the mammary gland of cows to protect their offspring against salmonellosis via locally produced specific IgA and IgM in colostrum. The method probably provided some protection in calves receiving colostrum from vaccinated cows, because they exhibited fewer clinical signs after the challenge and had a reduced excretion. However, the duration of excretion was similar to that of calves from unvaccinated dams.

The most promising vaccination results to date were reported by Mizuno et al. (2008) who investigated safety, in vivo behaviour and protective properties of oral and intramuscular vaccination with live attenuated *S*. Dublin-mutant N-RM25. Vaccination by either route significantly reduced clinical signs and faecal shedding, prevented the development of systemic infection and protected calves from a challenge, normally lethal, conducted within 14 days post-immunisation in calves less than six weeks old. Shedding of challenge bacteria, however, was not fully prevented in any of the groups. The authors concluded that intramuscular administration of N-RM25 was safer than oral administration in terms of the environmental contamination by the vaccine strain and provided better early onset protection in the young calves. The study was performed in Australia, but I have not been able to find out if this vaccine is commercially available.

Vaccination is not used for the control of *Salmonella* in livestock in Denmark for several reasons. A major reason is probably tradition, but also the fact that with live vaccines there is a concern about the environmental contamination. Although the bacteria used for live vaccines are usually harmless they are shed by the animals given the vaccines, and studies are needed to investigate the effect on other species that may be exposed to these bacteria. Furthermore, the vaccination trials presented above were based on repeated treatments of calves. This is an approach that is likely to be non-profitable for the farmer. Vaccinations for protection are unlikely to lead to an eradication of the infection, but may be useful in an outbreak situation where losses can be reduced until the transmission of the infection can be reduced. In addition it might be a useful tool in e.g. large herds that have difficulty breaking the transmission routes even with very good hygiene and management (Nielsen et al., 2012 **Paper IX**). Future simulation studies to evaluate the cost-effectiveness of vaccination procedures as part of control strategies are therefore highly recommended. However, documentation of vaccine efficiency has proven difficult to obtain from vaccine producers and published studies, so it will be difficult to parameterise the simulations accurately.

Preconditions for eradication

According to Yekutiel (1980) there are six technical, epidemiological, socio-economic, operational and/or administrative preconditions for the eradication of infectious diseases to be feasible and sensible:

(1) available knowledge and effective tools for breaking transmission;
(2) favourable epidemiological features of the disease;
(3) socio-economic importance of the disease;
(4) specific reasons for preferring eradication over control;
(5) adequacy of administrative, operational and financial resources;
(6) absence, or small extent, of adverse factors of human ecology.

Evidence has been presented in Chapters 2 to 7 that the required preconditions are present in Denmark to make eradication a recommended approach for *S*. Dublin in the cattle population, except for precondition 5 and 6. Having a large livestock industry and high standards for food safety animal health and welfare Denmark must be expected to be able to provide the administrative, operational and financial resources to eradicate *S*. Dublin, and the steps taken so far strongly indicate that this is the case. It is mostly a matter of priority, whether it will be done and how many resources are assigned to the task. The discussion is on-going about who should pay for the cost of control: the individual farmer, the industry as a whole, the consumers or the government. Precondition 6 is concerned mainly with the socio-ecological conditions that the eradication campaign imposes on people or which may be a hindrance to the success of the programme, such as an evacuation of people away from their homes, cultural habits or beliefs that counteract the eradication campaign. It is difficult to see how the Danish approach to the eradication of *S*. Dublin can be hampered by socio-ecological factors other than counteractive perceptions, attitudes and behaviours among some farmers (Kristensen and Jakobsen, 2011) and some local veterinarians and cattle advisors.

Chapter 8
Conclusions and perspectives

S. Dublin is a bacterial infection in cattle. It is host-adapted to cattle, so the sporadic cases seen in other species are usually traced back to a cattle or beef source. The infection leads to increased morbidity, mortality and substantial production losses in many of the infected cattle herds. In addition there are up to 50 annual human cases of salmonellosis recorded as caused by *S*. Dublin, and these are mostly attributed to consumption of contaminated imported as well as domestic beef in Denmark. Human cases are characterised by systemic disease and are difficult to treat. Patients who are ill with or have been ill with *S*. Dublin infection have a significantly higher risk of dying than patients who have been ill from other *Salmonella* serotypes.

The work presented in this thesis provides evidence that the required technical, epidemiological and economic preconditions for eradication of *S*. Dublin exist in Denmark. Furthermore the work has contributed considerably to secure sufficient and solid research-based evidence for effective control methods against *S*. Dublin in cattle herds, without the use of vaccination or expensive culling programmes.

In fact, Denmark is well on its way to eradication of *S*. Dublin in several regions of the country (e.g. Bornholm, Zealand, Funen, the Islands and East Jutland are very close to zero infection). Furthermore, a reduction in the apparent prevalence from 26% in 2002 to 7.3% in August 2012 among the dairy herds and an encouraging downward trend in the apparent prevalence over the first half of 2012 seem promising. However, it is uncertain that the goal of complete eradication of *S*. Dublin from the Danish cattle population will be reached before the end of 2014.

Several questions and requests for more knowledge about specific estimates and science-based solutions have been raised since 2002, and the provided answers and solutions have been used widely in the adjustments of the surveillance programme and the planning of the intensified control and eradication programme from 2007. Many of these questions and requests were phrased as research hypotheses in this thesis and answered through the studies presented in the accompanying papers. A review paper on the pathogenesis and the diagnosis of *S*. Dublin was included in the thesis (Nielsen, 2013 **Paper I**), to support investigations of epidemiology and infection dynamics, and to help when designing and evaluating surveillance and control programmes.

Within-herd prevalence of S. Dublin

The within-herd prevalence of *S*. Dublin varied tremendously from 0% to 70% among endemically infected dairy herds, and was generally more stable in young stock and adult cows than in calves <180 days. Faecal culture prevalence was generally low (i.e. the highest faecal culture prevalence was 5.4% in dairy calves during the period of December to February), and faecal shedding was generally much more infrequent in adult cattle, except in a few herds where faecal shedding was exclusively found in adult cows. The age and the season affected the within-herd prevalence (Nielsen, 2013 **Paper II**). The seroprevalence was generally highest in the calves between 3 and 6 months old, i.e. on average it was 27-42%; from June to November it was below 25% and from December to May is was above 30%, on average. In young stock the seroprevalence stayed around 25-30% for all seasons. The seroprevalence was associated with the occurrence of positive faecal

cultures in calves and adult cows, but not in young stock. This suggests that calves and adult cattle are the best indicator groups for serological detection of infectious herds in herd classification procedures.

The large variations in *S*. Dublin prevalence within infected dairy herds was found to be explained by the infection being highly dynamic, meaning that it spreads rapidly within susceptible groups of cattle and only leads to short-term resistance in previously infected animals (Nielsen et al., 2012 **Paper IX**). Furthermore, many age groups in dairy cattle herds are very dynamic with a more or less continuous in-and out-flow of cattle. The basic reproduction parameter, R_0=2 estimated for *S*. Dublin in the calves <180 days under Danish dairy herd conditions basically means that infectious calves on average will spread the infection to two other calves in a fully susceptible population during its own infectious period (Nielsen et al., 2007 **Paper III**). While this does not sound like much, the fact that calves are often kept in small groups and that the bacterial excretion from a newly infected calf can start already the day after it becomes infected, all calves in a common pen or calf hut can easily become infected, infectious and resistant within the time they are housed together (Nielsen et al., 2007 **Paper III**; Nielsen et al., 2012 **Paper IX**). If good separation of transmission pathways between calves in different pens is not in place, it often leads to transmission through the whole section; the background risk of spreading infection between housing sections in the herd was demonstrated to be important for the tendency of *S*. Dublin to become persistent in the herd by simulation modelling (Nielsen et al., 2012 **Paper IX**). In fact, management factors leading to improved hygiene and fewer contacts between individual animals and groups of animals in dairy herds were found to be the most critical parameters determining the duration of the infection, the epidemic size, the probability of elimination, and the consequences of introduction of the infection. The effect of this parameter increased with increasing herd size, because there are more new susceptible animals being introduced to the herd and to subgroups in the herd, the larger the herd is (Nielsen et al., 2007 **Paper III**; Nielsen et al., 2012 **Paper IX**). It can be argued that a high prevalence of previously exposed adult cows may have a dampening effect on the number of new infections among cows, which may consequently lead to fewer abortions and clinical cases in the cow barn. The benefit in having herd immunity, is thus outweighed by the effect of the infection on milk yield in cows, and by elevated morbidity and mortality in calves in endemically infected herds (Nielsen et al., 2010; Nielsen et al., 2012 **Paper IX**; Nielsen et al., 2013 **Paper X**; Nielsen et al., 2012a).

The prevalence of faecal culture positive cattle delivered to slaughter was on average 1.5%. However, the infected animals were highly clustered within particular herds in which the within-herd prevalence varied between 5% and 25% (Nielsen et al., 2011 **Paper IV**). For comparison the within-herd true prevalence estimates from a study using Bayesian estimation varied between 21% and 49% infected cattle, i.e. 2-4 times higher (Nielsen et al., 2011 **Paper V**). The underlying condition may, however, be a state of the infection that is less infectious than that detected only by faecal culture. The estimated prevalence levels were similar to those found in endemically infected dairy herds. These studies emphasised the importance of taking into account diagnostic sensitivity and specificity of the used testing procedures not to underestimate the prevalence. Using the Bayesian approach has an added benefit in that it can estimate the diagnostic accuracy of the used tests simultaneously and these may not always be known prior to performing the study. Together the two studies highlight the importance of determining a target condition of interest, when designing the study. Otherwise, it can be difficult to get unbiased results relevant

for the purpose of the study, e.g. to evaluate food safety or herd classification for a surveillance programme aiming at monitoring and ensuring progress over time at the national level.

Herd level prevalence of S. Dublin

The estimated true herd level prevalence of *S*. Dublin infected veal calf herds delivering more than 100 cattle to slaughter per year was between 34% and 57% in the study based on Bayesian estimation of the prevalence (Nielsen et al., 2011 **Paper V**). This was markedly higher than the 18% estimated in the study relying only on bacteriological cultures (Nielsen et al., 2011 **Paper IV**). At the time the study was performed, the prevalence of antibody positive veal calf herds of the same size was between 35% and 40% (Chapter 3) which corresponds well to the estimates from the Bayesian study. It was also found in that study that serology as used in the national surveillance programme for herd classification was sufficient for estimation the prevalence in veal calf herds (Nielsen et al., 2011 **Paper V**). Most importantly although not surprising, it was found that the more cattle, veal calf herds purchased from test positive dairy herds, the higher the risk was that they would send infectious cattle to slaughter. In conclusion, a substantial proportion of veal calf herds pose a risk to food safety by delivering animals with *S*. Dublin in the intestinal contents, and that this risk can be reduced by avoiding the purchase of cattle from infected dairy farms. This finding is important and might useful to develop new control initiatives or be implemented into the legislation. In the field study, there were large veal calf herds that did not purchase calves from test positive suppliers and that had zero test positive serological and faecal samples in the slaughtered cattle, so it is not only recommendable, but also practically feasible.

Very few reliable studies on herd level prevalence of *S*. Dublin in dairy herds were identified in the literature, and most of those that were available underestimated the true prevalence, because they were mainly based on faecal cultures or other sampling methods that lacked sensitivity. Several studies and further calculations in Chapter 3 of this thesis have contributed considerably with detailed knowledge about the development in the herd level true and apparent prevalence among Danish dairy herds, including regional and national changes over time (Warnick et al., 2006 **Paper XIV**; Nielsen et al., 2007 **Paper VI**). In all regions, the herd level prevalence has been reduced by at least 50% of the start-out prevalence in 2002. However, analysis of incidences and incidence risks based on uniquely large and longitudinal datasets from the national surveillance programme revealed that new infections still occur, meaning that the surveillance and control programme are not fully successful in preventing spread of infections between herds in their current structure (Nielsen and Dohoo, 2012 **Paper VII**). In that study it was found that the most important predictors for introduction of new infections or re-infections with *S*. Dublin include purchase of animals from test positive dairy herds, high local prevalence in a 5 km radius around the herd, herd size and factors indicating poor hygiene or biosecurity such as high somatic cell counts in the bulk-tank milk. The study also showed that the incidence risk in previously infected herds is markedly higher than in the herds that have not been infected up to 3 years after they become test-negative following clearance of infection. This emphasizes the importance of ensuring high external and internal biosecurity procedures even after a herd has reached Level 1 (test negative) in the programme.

Risk factors

Few risk factor studies specific for *S*. Dublin were identified in the literature (Vaessen et al., 1998; van Schaik et al., 2002). Most available studies were on *Salmonella* infections in general in cattle

herds (Warnick et al., 2001; Fossler et al., 2005a; Fossler et al., 2005b). Several risk factor studies were therefore included in this thesis using different approaches and angles to extract information useful to design surveillance and control programmes and to provide evidence-based advice to farmers and farmers' organisations about potential control options in the herds.

The most consistent risk factors associated with introduction or occurrence of *S*. Dublin in cattle herds were purchase of cattle from infected (or test positive) farms, herd size and local prevalence (Nielsen et al., 2007 **Paper VI**; Nielsen et al., 2011 **Paper IV**; Nielsen and Dohoo, 2012 **Paper VII**; Nielsen and Dohoo, 2013 **Paper VIII**). The effect of local prevalence has been confirmed in another study using spatiotemporal analysis, and distances between 1.5 to 8.3 km from an infected herd were found to increase the risk of becoming infected for neighbouring herds (Ersbøll and Nielsen, 2008). Local spread may occur both directly and via more diffuse routes that are still unclear. A newly identified risk factor indicating good within-herd level of biosecurity was also found important in preventing introduction and persistence of infection, e.g. low BTM somatic cell counts (Nielsen and Dohoo, 2012 **Paper VII**; Nielsen and Dohoo, 2013 **Paper VIII**). The risk of introduction or re-introduction of *S.* Dublin was found to be markedly higher for up to approximately 3 years after a herd had been infected and became test negative than for herds with no prior history of infection (Nielsen and Dohoo, 2012 **Paper VII**). Herds that were enrolled in a voluntary control paratuberculosis programme had markedly shorter durations and consequently lower risk of remaining persistently infected in high prevalence regions than herds not enrolled in the programme. There was no difference for herds enrolled and herds not enrolled in the programme under low prevalence conditions. This might suggest that herds enrolled in the programme had superior external biosecurity and that this helped prevent reinfections with *S.* Dublin (Nielsen and Dohoo, 2013 **Paper VIII**). It also suggests that there a synergetic effects of running control programmes for multiple diseases simultaneously, at least when their transmission routes overlap.

Organic farming on the other hand, was found to be associated with a higher tendency for herd infections to persist in the herd, probably due to management practices allowing more frequent and longer contact between animals in the herd (Nielsen et al., 2007 **Paper VI**; Nielsen and Dohoo, 2013 **Paper VIII**). Organic farming was not associated with increased risk of introduction of *S*. Dublin infection to the herds (Nielsen et al., 2007 **Paper VI**; Nielsen and Dohoo, 2012 **Paper VII**), so the external biosecurity is likely to be similar to that of conventional herds. Finally, there was evidence that control of *S*. Dublin was stimulated by centrally organised initiatives (Nielsen and Dohoo, 2013 **Paper VIII**). These studies were the only large scale studies available that systematically evaluated duration and risk factors for introduction and recovery, plus time-dependency of the risk factors of *S*. Dublin in dairy herds.

Infection dynamics within dairy herds

Duration of infectiousness in young calves was estimated to be on average 17 days (median=10). However, the range was wide (3 to 68 days). Isolations of *S.* Dublin bacteria were made in all ages of calves including few days old calves (Nielsen et al., 2007 **Paper III**). In the same study, time to seroconversion after onset of shedding was estimated from repeated antibody measurements in serum samples to be on average 36 days (range 11-67 days)(Nielsen et al., 2007 **Paper III**). Thus, there was coherence between the results from experimental studies and this field study on important elements of transmission dynamics.

The simulation results from the 'Dublin-Simherd' simulation model showed that the hygiene level was highly influential on the probability of spread of infection within the herd, duration of infection and epidemic size. The herd susceptibility level was also influential, but not likely to provide sufficient prevention and control of infection on its own. In addition, herd susceptibility is more difficult to change markedly through daily management unless vaccination is available. The use of vaccination to control *S*. Dublin was discussed based on previous experimental and field studies, and it was concluded that vaccination, despite the widespread use in several countries, is not likely to be an effective, nor a cost-effective solution to control of *S*. Dublin. However, further simulation of different control scenarios including vaccination is recommended. Herd size did not as such affect the probability that infection would take off and start spreading upon exposure, but the larger herd sizes required correspondingly better management and housing that optimised hygiene and reduced susceptibility of the cattle to shorten durations of infection in the herd and to increase the probability of extinction. The infection persisted in most of the large herds (400 cow stalls) in the simulations, unless the hygiene and herd susceptibility levels were set to a relatively high values, which implies that there might be an added benefit of vaccination under such specific conditions.

Sensitivity analyses of 24 alternative scenarios showed that a 'super shedder' state was not required to mimic real life infection dynamics, which may indicate that this state does not exist or only very rarely occurs. However, a persistent carrier state was required to mimic the infection dynamics and persistence patterns known from field studies (Nielsen et al., 2012 **Paper IX**).

Test-strategies and surveillance

It was hypothesised that animals with persistently high *S*. Dublin antibody levels will excrete bacteria in faeces more frequently than animals with persistently low or fluctuating antibody levels. They were therefore assumed to pose a higher risk of spreading the infection in the herd. Carriers probably do exist – as both literature and the Dublin-Simherd simulations suggest. However, longitudinal studies of all cattle in 14 endemically infected dairy herds (Nielsen, 2013 **Paper XII**), and an experimental study in which it was attempted to provoke adult cattle with persistently high antibody levels to excrete bacteria (Lomborg et al., 2007 **Paper XIII**) suggested that repeated antibody measurements used for carrier detection are not recommendable as a key control element in cattle herds. Animals with repeatedly high antibody levels did not present a clearly elevated risk of shedding *S*. Dublin bacteria compared to cattle with recent increased antibody levels or animals with fluctuating antibody levels, except perhaps in heifers below 1-2 years old. In one study a high seroprevalence was found to lower the likelihood of cows and heifers getting culled in 10 dairy farms that participated in a control field study (Nielsen and Dohoo, 2011 **Paper XVI**). It may still be useful to find cattle with high antibody levels and cull them towards the end of a successful control programme where there will be very few of these left in the herd, because they tend to keep the bulk-tank milk level of antibodies up.

The classification system used in the Danish *S*. Dublin surveillance programme leads to few false negative herds (~1% at an underlying prevalence of 15% infected herds). The false positive classifications were more common (~20% at 15% true prevalence) mainly due to antibodies remaining in bulk-tank milk after dairy herds have recovered from the infection (Warnick et al., 2006 **Paper XIV**). In the future, the surveillance programme classification can be improved by adding an early warning component to reduce the false negative herd classifications, and by

performing follow-up testing in new test positive herds. In practice this could be done by requesting a sample of 8-10 blood samples from calves for ELISA testing or to request faecal samples be taken to also get estimates of the occurrence of potentially cross-reacting *Salmonella* serotypes in the cattle herds.

The accuracy of the herd classification testing scheme for non-dairy herds was evaluated for two scenarios: 1) the current where the classification of test positive is based on one sample ≥50 ODC%, and 2) an alternative scenario in which two samples ≥50 ODC% would be required to deem the herd test positive.

Moving to the alternative scenario from the current would increase the herd specificity (HSp) from around 60-65% to 90-100%. The herd sensitivity (HSe) would be reduced from the current 80%-100% to 45-90% depending on herd size and within-herd prevalence. In other words, it would lead to fewer false positive herd classifications, but would only be sensitive for detection of high prevalence herds. If the purpose of testing is to follow the development in national prevalence over time or if false positive herd classifications have large consequences for the farmers, it might be worth to use the criterion that would ensure a high HSp. This criterion, however, does not combine well with reduced sample size in small herds, because HSe quickly becomes markedly lower when n is below 7. Careful selection of herd classification criteria in relation to the purpose of the programme of is as important as the choice of tests as an instrument of *S*. Dublin surveillance and control.

Control and eradication

A study that modelled different control scenarios in 2006 and 2007 accompanies this thesis (Jordan et al., 2008 **Paper XI**). It showed that the most effective improvement to the programmes would be to include initiatives that would reduce the duration of infection in the infected herds. Consequently, industry-driven centrally organised and financially supported initiatives were started in September 2007 and continued throughout 2008 and 2009 involving different field research projects and a control campaign based on farmer experience groups and direct consultancy of local advisors, which was found to work as anticipated (Nielsen and Dohoo, 2013 **Paper VIII**). In 2010 the centrally organised voluntary efforts were reduced and replaced with new legislation involving official veterinary supervision by the regional veterinary authorities. This new legislation mainly imposes firmer movement restrictions on herds with high antibody levels (Level 2R). This combined with an industry-strategy to leave it more up to individual farmers to decide what to do about *S*. Dublin in their herds may be an explanation for the worrying stagnation in the prevalence around 9-10% between 2010 and 2012.

Other studies have pointed out that positive attitudes and intentions of farmers to improve biosecurity to combat endemic and zoonotic diseases are not always easy to find (Heffernan et al., 2008; Ellis-Iversen et al., 2010; Kristensen and Jakobsen, 2011) even though hygiene and other external and internal biosecurity measures have proven essential for effective control and eradication of *S*. Dublin (van Schaik et al., 2002; Nielsen et al., 2007 **Paper VI**; Nielsen et al., 2011 **Paper IV**; Nielsen and Nielsen, 2012 **Paper XV**; Nielsen et al., 2012 **Paper IX**; Nielsen and Dohoo, 2012 **Paper VII**; Nielsen and Dohoo, 2013 **Paper VIII**; Nielsen et al., 2012b). As pointed out by Ellis-Iversen et al. (2010), there are large differences between farmers concerning which control initiatives will have the strongest effect on their personal motivation to adopt control measures. Sometimes it requires personal motivators such as the local veterinarian, whereas other farmers

are motivated by financial rewards or penalties, consumer demands and official control programmes. It is therefore strongly recommended that decisions about the control strategies for *S*. Dublin in cattle herds involve initiatives to cover the whole range of motivators in order to reach the goal of eradication of *S*. Dublin from the Danish cattle population by end of 2014. This recommendation is also important for other countries with similar cattle production systems and beef and milk markets, and who wish to control *S*. Dublin, even when eradication is not the goal.

Estimation of the consequences of *S*. Dublin in infected dairy herds at the level of detail provided in Nielsen et al. (2013 **Paper X**) showed that there are ample arguments for initiating control efforts in *S*. Dublin infected herds to avoid long-term losses, even if the required actions involve some investments and other control related costs. The systematic stepwise approach presented by Nielsen and Nielsen (2012 **Paper XV**) provided a demonstration of a cheap and effective method to control of *S*. Dublin in dairy herds, and it has also been used in an adjusted form in veal calf herds.

In my opinion

- Lack of highly sensitive tests are unlikely to impede the eradication programme, because currently available diagnostic tests (ELISA) for antibody measurements in BTM and individual animal samples can be combined to obtain good sensitivity and reasonable specificity already a few weeks after the start of an outbreak of *S.* Dublin at low costs, if used in the most optimal ways in the programmes.
- The ELISA-tests have specificity issues both due to cross-reactions with other *Salmonella* serotypes and in particular because it takes time for the antibodies to disappear from a herd after *S.* Dublin-infection has been removed. This problem can be solved at herd level using individual testing and culling of cows with high antibody levels towards the end of an eradication period.
- Widespread test-and-cull procedures, however, are not the answer to *S*. Dublin eradication. Management actions to reduce the environmental infection load and to prevent transmission of bacteria between animals are essential, if eradication of the infection from the cattle population is the goal. In particular, good calving practices and good management practices of newborn and young calves are essential. The significant economic losses associated with *S*. Dublin in infected herds leave financial room for the important changes in management and hygiene. However, simulation of optimal control strategies under different circumstances is recommended. 'Dublin-Simherd' allows for herd specific simulations, which in some cases, is a helpful advisory tool.
- Surveys to investigate the distribution of *Salmonella* serotypes in the Danish cattle population today and onwards are recommended to avoid unpleasant surprises such as a shift towards a higher proportion or an increased occurrence of *S*. Typhimurium or other serotypes, and decisions will have to be made whether other *Salmonella* serotypes should be included in the surveillance and/or eradication programme.
- Danish cattle herds are today tested and classified into surveillance levels, so that farmers are able to obtain good protection of their herds against infection when they need to purchase animals, if they choose to use the available systems. This was made possible by the extensive research into development and evaluation of diagnostic test-strategies and surveillance procedures for herd level diagnosis of *S*. Dublin in cattle herds and by means of the rigorous system for recording of animal movement and other animal and herd characteristics in the

Danish Cattle Database. This reasonably straightforward classification system was readily understood by the farmers at initiation of the surveillance program, and had a dramatic change of the trade patterns just a few months into the programme.

- Denmark has provided an ideal 'laboratory' for studying *S*. Dublin epidemiology as well as the effect of large-scale control strategies, which can be adapted to other countries. Denmark has the advantage of being a small country with all cattle owners organised within the same organisation, the Knowledge Centre for Agriculture, Cattle, associated with the Danish Agriculture & Food Council, which again has good collaboration and fairly direct contact to politicians and the Danish Veterinary and Food Administration. Furthermore, there is excellent access to data from all herds in the Danish Cattle Database. All cattle in Denmark are ear-tagged within the first days after birth and all movements between herds are recorded. Movement of animals is already part of the surveillance program for *S*. Dublin, but it is likely that this can be used in more optimal ways to limit transmission of *S*. Dublin between herds in the future for instance by restricting the movement out of the infected herds or the movement from the high-prevalence regions into the low-prevalence regions.

Future perspectives

Suggestions for improvements to the *S*. Dublin control strategies and suggestions for further research include:

- Investigation and implementation of risk-based surveillance methods to lower the cost of surveillance because active surveillance activities are aimed at high-risk areas or herds. For instance, herds that have been classified in Level 1 for many years and are located in low-prevalence regions could be tested less frequently, while herds with recent infection, infected neighbours, risky trade patterns and so on, could be tested more frequently or placed in an "uncertain level" until they have provided better evidence of the status of their herd.
- Early-warning systems might aid in fast detection and reduce the duration of infection if management to block transmission routes are started quickly after an outbreak/new infection. A part of this can be to improve today's passive surveillance system. If reporting of new outbreaks was made more attractive, this could improve early warning and reduce economic losses to everyone's benefit.
- Further investigations of the benefit and potential to implement multiple-disease control programmes.
- Studies to determine the prevalence of *S*. Typhimurium-infected cattle herds and whether there is a risk that this infection may increase in prevalence, as *S*. Dublin decreases. This phenomenon has been observed in Great Britain and it would be problematic to eradicate one serotype of *Salmonella* just to have to deal with another.
- Trace-back and trace-forth systems are used in Sweden where there are today very few outbreaks per year. Such methods should be considered as a part of the outbreak prevention strategy when Denmark reaches low prevalence.
- Improved slaughter procedures or restrictions for cattle from infected herds when prevalence becomes lower, e.g. only slaughter of Level 2 herds on Fridays. This can improve food safety, but also work as a motivator to control the infection in the herds.
- Studies to determine if human *S*. Dublin cases can be avoided through improved biosecurity elsewhere in the farm-to-fork-chain, than the primary production.

- Studies to determine which import restrictions would help to reduce the number of human *S*. Dublin-cases and human *S*. Dublin-case fatalities from imported meat once the infection is ultimately eradicated from Danish cattle.
- Assessment of the socio-economic consequences and benefits of the eradication campaign at sector and national level, including spin-off benefits on occurrence of other diseases, animal health and welfare, and production.

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PART 2: ACCOMPANYING PAPERS

Index for accompanying papers

PAPER I

Review of pathogenesis and diagnostic methods of immediate relevance for epidemiology and control of *Salmonella* Dublin in cattle

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Published in
Veterinary Microbiology 2013, 16: 1-9

(This paper constitutes Chapter 2 in the thesis and is therefore not included here together with the other accompanying papers.)

PAPER II

Within-herd prevalence of *Salmonella* Dublin in endemically infected dairy herds

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Within-herd prevalence of *Salmonella* Dublin in endemically infected dairy herds

Liza Rosenbaum Nielsen

Summary

In this study within-herd prevalence of *Salmonella* Dublin was investigated in three age groups (calves, young stock and adult cows) at five herd visits with 3 month intervals of 14 endemically infected dairy herds. A total of 10,162 paired faecal cultures and antibody measurements were used to calculate the age and temporal dynamics of seroprevalence and prevalence of positive faecal cultures. Faecal culture prevalence was generally low. It was highest (5.4%) in calves during the period of December to February. Seroprevalence varied from 0% to 70% between herds, but was generally more stable in young stock and adult cows than in calves. Hierarchical mixed model results showed that seroprevalence was associated with the bacteriological status in calves and cows, but not in young stock. These results can be used to develop and validate theoretical infection dynamics models and to design effective control programmes for *Salmonella* Dublin in dairy herds.

Introduction

Salmonella enterica subsp. e*nterica* serovar Dublin (*S*. Dublin) is a gastrointestinal bacterial infection of concern in intensive cattle rearing farms because it leads to increased morbidity and mortality as well as production losses (Richardson and Watson, 1971; Nielsen et al., 2010; Nielsen et al., 2012b). In order to design effective control programmes, good estimates of within-herd prevalence of infection are required (Veling, 2004; Nielsen and Nielsen, 2011). Furthermore, within-herd prevalence estimates are needed for development and validation of theoretical models of *S*. Dublin infection dynamics (Xiao et al., 2005; Lanzas et al., 2008; Nielsen et al., 2012a). There are however, very few published studies available that provide good insight into within-herd prevalence and dynamics of S. Dublin, in particular for persistently infected cattle herds.

Veling et al. (2002) investigated seroprevalence in 79 dairy herds 2 to 4 months after confirmed outbreaks of *S*. Dublin. The seroprevalence varied between 10% to almost 60%; however averaged seroprevalence estimates above 30% across all herds were only found in calves between 3 - 7 months old. In adult cattle the seroprevalence was on average 12%. The authors of that study also reported that the seroprevalence in young stock did not differ between infected herds with or without clinical signs, indicating that serology of young stock is a good indicator of subclinical *S.* Dublin infection in the herd. However, a subsequent study in some of the same herds showed large variation in prevalence between herds, i.e. from 0 to 70% in both young stock and adults (Veling, 2004).

In another study done in California, of a large persistently infected dairy herd with clinical problems associated with *S*. Dublin, showed the prevalence of seropositive adult cows was 3.5%, whereas in calves it was 52%. In that study, 11% of the calves were found to be faecal shedders of *S*. Dublin bacteria (House et al., 1993). However, neither herd size nor management reported in that study were representative of Danish dairy herds, so a field study was performed to gain more knowledge about the occurrence of *S.* Dublin within endemically infected dairy herds in Denmark.

The objective of this study was to investigate age- and time-related dynamics of within-herd seroprevalence and faecal excretion in endemically infected dairy herds.

Materials and methods

Selection of herds and sampling

In year 2000, a total of 14 dairy herds were selected to participate in a field study on the basis of them having high bulk-tank milk ELISA results, i.e. above 50 background corrected optic density values (ODC%) (Andersen et al., 2000; Nielsen, 2003). Herd size varied between 38 and 154 lactating cows. *S*. Dublin was isolated from faecal samples of all of these herds at least once during the study period from the beginning of 2000 to the beginning of 2002 (Nielsen and Ersbøll, 2005), with indications of the herd being endemically infected throughout the project period (i.e. continued serological responses in all age-groups of cattle and bulk-tank milk throughout the study period). Each of the 14 herds was visited five times – except one that was visited four times – within a time frame of approximately 3 month intervals. At each visit, blood samples were collected from all accessible calves, young stock and dry cows; and milk samples were collected from all lactating cows at the morning milking for serological analysis. Faecal samples were collected rectally from all accessible animals and placed into marked faecal transport containers with snap cap (549263 NUNC A/S, Roskilde, Denmark), aiming at getting at least 50 g from each animal.

All samples were transported directly to the Danish Cattle Health Laboratory (DCHL) in Ladelund, and kept below 5°C until analysis could be performed within a few days of the samples' arrival. At the laboratory faecal samples were pooled 5 at a time using 5 g per sample. This was then mixed in a 25 g pool before analysis. The blood samples were spun to extract the serum fraction for analysis.

Bacteriological culture method

Pooled faecal samples were examined at DCHL for the presence of *Salmonella* bacteria by mixing the 25 g of faecal material in a 225 ml peptone buffer and left for pre-enrichment at 37°C for 18-24 hours. A volume of 0.1 ml of the test material was added to Modified Semi-solid Rappaport Vassiliadis Medium Base (MSRV-agar) plates and 1 ml of the test material was put into 9 ml of selenite-cystine broth and incubated for 18 - 24 hours at 41.5°C. Material from the selenite-cystine tubes was inoculated on modified Brilliant-green Phenol-red Lactose Sucrose agar (BPLS-agar) plates and incubated at 37°C for 18 - 24 hours. Positive test results from MSRV were inoculated onto BPLS-agar plates and confirmed using Triple Sugar Iron agar-tests and Lysine Iron-agar tests. Serotyping and confirmation of positive isolates were conducted at the Danish Veterinary Institute (today the National Food Institute at the Danish Technical University in Copenhagen).

If the pool was found to be positive for Salmonella, then the individual samples would be cultured using 25 g of faecal material to try to identify those animals that were positive in the pool. The diagnostic sensitivity of this faecal culture procedure has been evaluated to be approximately 6-14% in subclinically infected cattle (Nielsen et al., 2004).

Antibody measurements by ELISA

The serum *S*. Dublin ELISA that was used in this study, was performed at DCHL as slightly modi-fied from a previously described ELISA method (Hoorfar et al., 1994) and described in detail in Nielsen and Ersbøll (Nielsen and Ersbøll, 2004). In short, an O-antigen based *S*. Dublin lipopolysaccharide

(LPS) preparation produced at the Danish Veterinary Institute in Copenhagen was used to coat microtitration plates. Sera were diluted 1:200 and added to micro-titration plate wells in duplicates. Known positive and negative reference sera were added in quadruplicates. The plates were incubated overnight at 4°C, and washed 3 times. For detection of immunoglobulins, affinity purified horseradish peroxidase-labelled goat anti-bovine IgG (H+L) conjugate was added. Following incubation for 1 hour at 37°C the plates were washed 3 times. Substrate and indicator solution was added to the wells and incubated in the dark at room temperature for 10-20 minutes. The reaction was then stopped when the optical density of the positive reference wells was visually evaluated to be approximately 2.000 optic density values (OD). The OD was read at 492 nm and 620 nm as reference using an ELISA plate reader. Plates were considered valid if the 4 negative reference wells had an average OD of less than 0.300, and the 4 positive reference wells had an average OD of 1.200-2.500. An ODC%-value, which is a background corrected proportion of the test sample OD to a positive reference sample, was calculated as follows:

$$ODC\% = \frac{\left(\overline{OD}_{sample} - \overline{OD}_{neg\ ref}\right)}{\left(\overline{OD}_{pos\ ref} - \overline{OD}_{neg\ ref}\right)} * 100\%$$

where $\overline{OD}_{sample}$ is the mean value of two test wells, $\overline{OD}_{negref}$ and $\overline{OD}_{pos\ ref}$ are the mean of ELISA plate readings of four test negative and test positive reference wells, respectively.

Serum and milk samples with ODC% > 50 were considered seropositive in the consecutive calculations for within-herd and within-age group prevalence estimations. At this cut-off, the sensitivity of the ELISA has previously been estimated to be 0.16 - 0.26 for calves below 100 days old, 0.66 - 0.88 in calves and young stock from 100 to 300 days old, and 0.50-0.68 in cattle above 300 days old. The specificity was estimated to 0.93 - 0.98, 0.93 - 0.98, and 0.88 - 0.91 for the same age groups, respectively (Nielsen et al., 2004).

Statistical analyses

SAS® version 9.2 (SAS Institute Inc., USA) was used for the data management as well as the descriptive and statistical analyses. Within-herd prevalence of seropositive and bacteriologically positive animals was calculated for rolling intervals of age across all herds. Intervals of the serological prevalence contained 200 observations. Due to the low number of faecal culture positive animals, the intervals used for descriptive statistics of the bacteriological results contained 500 observations in each interval. For each interval, 95% confidence limits were calculated.

For further analysis, the dataset was split into three age groups based on typical management and housing structures in Danish dairy herds: Calves 0-180 days old (usually housed in calf barns with single housing followed by small groups of calves), young stock 181 days to 2 years old (growing and breeding heifers often kept in larger groups) and adult cattle above 2 years old (adult heifers close to calving or cows). The correlation between seroprevalence and faecal culture-positive prevalence within each age group and across all age groups was investigated using Spearman's correlation. Furthermore, the correlation between the faecal culture prevalences at one visit and the seroprevalences at the next visit for each age group and across all age groups were investigated using Spearman's correlation, because it might be expected that the increase in seroprevalence would be delayed by at least two to four weeks compared to the point in time of the shedding of the bacteria (Da Roden et al., 1992; Jordan et al., 2008; Robertsson, 1984).

Factors affecting seroprevalence at each herd visit were investigated for each of the age groups using three hierarchical mixed models with seroprevalence as the outcome and season and bacteriological status of the age group on the giving visit date as potential risk factors in the model. Repeated sampling at herd level was taken into account in the analysis, and the herd was included as a fixed effect to be able to determine the actual predicted seroprevalence for each herd and level of significant predictors. Two-way interactions between predictors were tested in the models. Predictors and interactions were considered significantly associated with the outcome if the *P*-value was below 0.05.

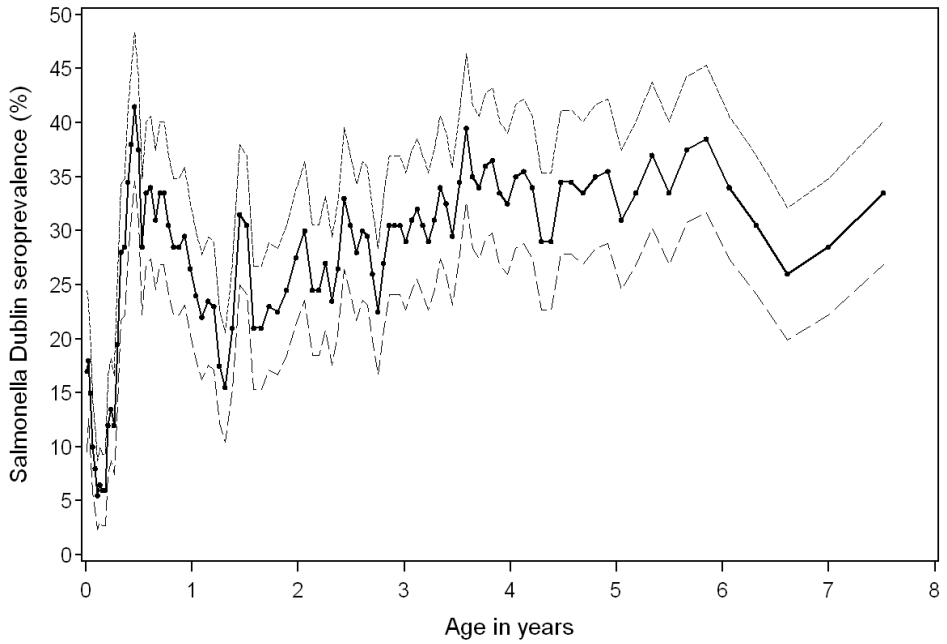

Figure 1 *S.* Dublin seroprevalence in 14 endemically infected dairy herds tested repeatedly during 2000-2002. The solid line shows the mean seroprevalence and the dashed lines the 95% confidence interval. The various points represent average age in rolling intervals which each contained 200 observations.

Results

There were a total of 10,162 observations with paired faecal cultures and ELISA results from all ages of cattle in the 14 study herds. Table 1 shows the distribution of seropositive and faecal culture-positive cattle in each season stratified by age groups across the 14 dairy herds. In the descriptive statistics and univariable analysis, the seroprevalence was lower (below 25%) in summer and fall than in spring and winter (above 30%) for calves; whereas for young stock the seroprevalence stayed around 25-30% for all seasons. For cows, the seroprevalence varied significantly with season. The seroprevalence was lower in the fall than in spring, summer and winter. The dynamic changes of the seroprevalence with age across all study herds are illustrated in more detail in Figure 1, and examples of how different these patterns were for each herd are illustrated for two herds in Figure 2.

Table 1 Descriptive statistics of *S*. Dublin seroprevalence and faecal culture prevalence by season, in three age strata of 14 endemically infected dairy herds. *P*-values provided are from χ^2-tests of the effects of season on the prevalence in univariable analyses for each age strata.

Factors	Distribution of animals and *P*-values from univariable analysis of the effect of season within each age strata			
Age group, season and age distribution within each group (mean; 5th and 95th percentiles)	No. of observations	% seropositive (95%CI)	% faecal culture-positive (95%CI)	*P*-values serology / bacteriology
Calves				0.001 / 0.02
Spring (135; 95-177 days)	279	33 (27-39)	1.1 (0-2.3)	
Summer (132; 94-176 days)	132	19 (12-26)	2.3 (0-4.8)	
Fall (135; 93-177 days)	180	24 (18-31)	1.1 (0-2.7)	
Winter (131; 93-176 days)	185	37 (30-44)	5.4 (2.1-8.7)	
Young stock				0.25 / 0.08
Spring (435; 197-687 days)	1118	25 (22-28)	0.9 (0.3-1.5)	
Summer (386; 187-684 days)	244	26 (20-31)	2.1 (0.3-3.8)	
Fall (351; 197-625 days)	463	30 (26-34)	1.5 (0.4-2.6)	
Winter (439; 212-696 days)	792	27 (24-30)	2.3 (1.2-3.3)	
Cows				<0.001 / 0.07
Spring (4.0; 2.2-7.4 years)	2027	33 (31-35)	0.4 (0.2-0.7)	
Summer (4.1; 2.3-7.3 years)	1071	32 (29-35)	0	
Fall (4.1; 2.3-7.5 years)	1264	26 (24-29)	0.2 (0-0.5)	
Winter (4.0; 2.2-7.3 years)	1376	34 (32-37)	0.5 (0.1-0.9)	

The prevalence of positive faecal cultures differed between seasons in calves (varied from 1.1% in spring and fall to 5.4% in winter, *P*=0.02). In young stock and cows a similar tendency was observed (0.9% in spring to 2.3% in winter, *P*=0.08, for young stock) and (from 0% in summer to 0.5% in winter, *P*=0.07, for cows) in the univariable analyses. The dynamics of faecal culture prevalence with age are illustrated in more detail in Figure 3. Hence, faecal shedding prevalence was up to twice as high in calves as in young stock, and up to 10 times higher in calves than in cows. In contrast, the seroprevalence was generally at comparable levels in all age strata.

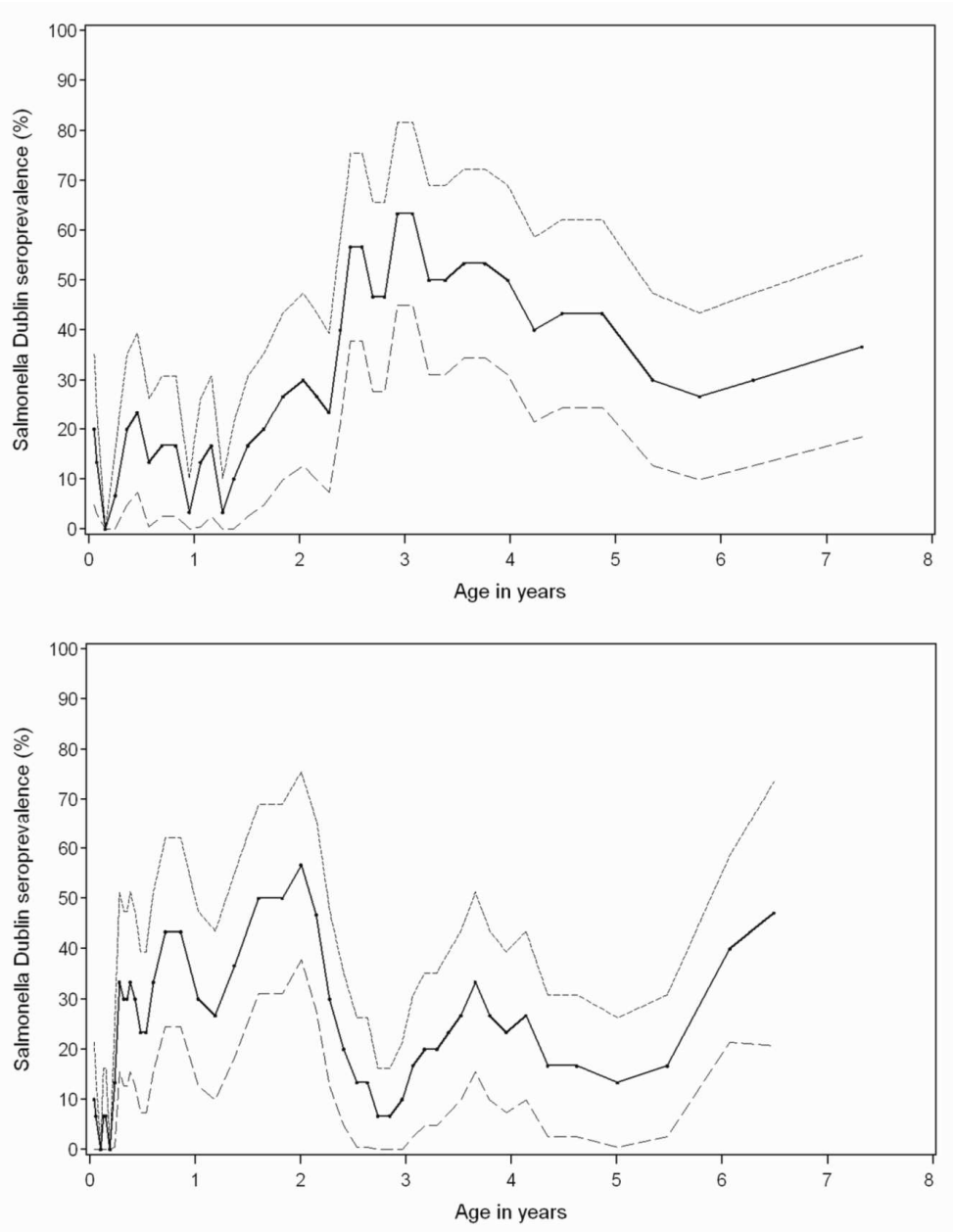

Figure 2 *S*. Dublin seroprevalence in two endemically infected dairy herds tested repeatedly during 2000-2002. The top graph illustrates the seroprevalence dynamics in a herd that was bacteriologically positive only among cows, and the bottom graph illustrates the same for a herd that was bacteriologically positive only among calves and young stock. The solid line shows the mean seroprevalence and the dashed lines the 95% confidence interval. The points represent average age in rolling intervals that each contained 200 observations.

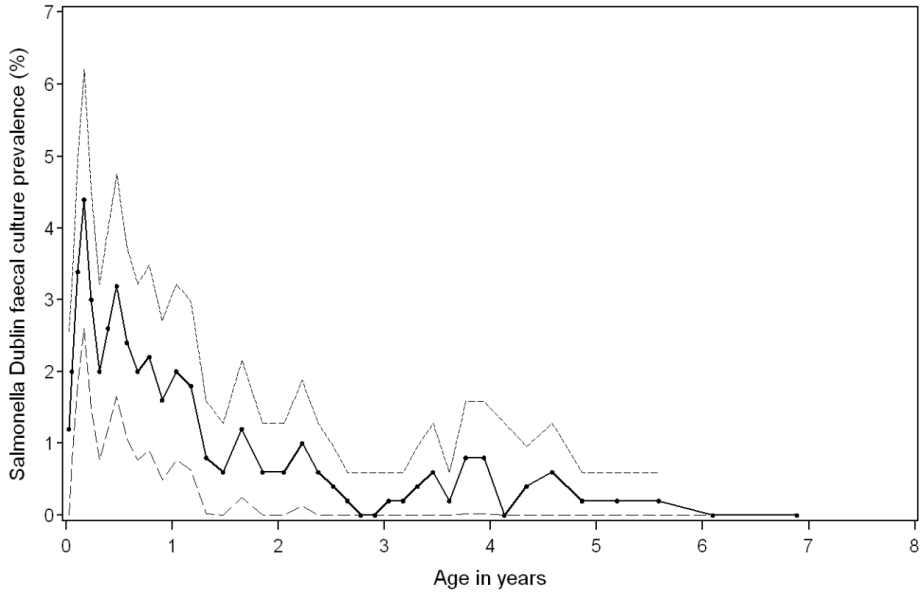

Figure 3 Prevalence of *S*. Dublin faecal culture-positive cattle in 14 endemically infected dairy herds tested repeatedly during 2000-2002. The solid lines show the mean prevalence in rolling intervals and the dashed lines the 95% confidence intervals. Points show average age of the rolling intervals that each contained 500 observations.

Spearman's correlation between seroprevalence and faecal culture-positive prevalence was generally low: at herd visit level (n=69 herd visits) the correlation coefficient ρ=0.197 (P=0.1). At age group visit level ρ=0.272 (P=0.02) in calves, ρ=0.285 (P=0.02) in young stock and ρ=0.372 (P=0.002) in cows. The Spearman's correlations between faecal culture prevalence and seroprevalence at the following herd visit (n=55) were similar to those found when comparing seroprevalence and faecal culture prevalence at the same visit, and graphic displays of seroprevalence vs. faecal culture prevalence at the same visit and at offset herd visits did not suggest specific patterns that would be of interest for further analysis (data not shown), so it was decided not to explore these patterns any further.

The model-predicted seroprevalence varied significantly between herds (i.e. up to 70% difference between the lowest to the highest predicted seroprevalence in calves). On average seroprevalence was 13.4% higher in calves, 7.4% higher in young stock and 11.3% higher in cows if *S*. Dublin was isolated from at least one faecal sample in the same age group at the same herd visit. Predicted seroprevalence for each herd and underlying bacteriological status is illustrated in Figure 4 for the three age groups. According to these models, the seroprevalence was not affected by season when taking into account the underlying bacteriological status of the age group.

Discussion

Using a large field data collection from 14 endemically infected dairy herds, this study provided new detailed information on the level and dynamics of faecal culture prevalence and seroprevalence for *S*. Dublin. The main finding was that the seroprevalence of *S*. Dublin varied tremendously between herds. Generally it was higher in herds with test positive faecal cultures at

the same herd visit. The seroprevalence was found to be significantly associated, but not highly correlated with the faecal culture prevalence in calves and cows. In young stock the correlation appeared to be significant in the Spearman correlation analysis. However, in the mixed model analysis, which took into account herd variation and repeated sampling of animals, there appeared to be no association between seroprevalence and faecal excretion of bacteria. The correlation between faecal shedding prevalence and seroprevalence at the following visit could have been higher than the correlation between the serology and the current bacteriology results for each visit due to the delay in serological responses upon infection. However, neither the descriptive nor the statistical analyses of these correlations showed any noteworthy difference in the correlations for any of the age groups or across the herds when taking into account the time delay between faecal shedding and serology (data not shown). This may be because these herds were endemically infected leading to continuous low dose exposure of the animals leading to little faecal shedding and fluctuating serology in all age groups. This is also reflected in the fact that the seroprevalence generally became more stable with increasing age as illustrated in Figure 1, hovering around 25% to 35% in adult cows. Surprisingly, this was markedly higher than the average seroprevalence of 3.5% reported by House et al. (1993) from a study of a large persistently infected dairy herd with clinical problems associated with *S*. Dublin, and the seroprevalence of 12% reported by Veling et al. (2002) for adult cows in recent outbreak herds. However, a subsequent study with some of the same herds showed large variation between herds with seroprevalence from 0 to 70% in both young stock and adults (Veling, 2004). These results have more similarities to the variations found in the present study (Figures 2 and 4). Together these studies indicate that the seroprevalence may be higher in adult cattle in persistently infected herds without clinical signs than in herds with outbreaks and clinical problems. Even though antibodies are not necessarily protective in individual animals (Chaturvedi and Sharma, 1981), but rather an indication of previous exposure which may have evoked cell-mediated immunity (Steinbach et al., 1993; Steinbach et al., 1996), a high seroprevalence may be an indication of a high level of herd immunity, which would explain the lack of clinical signs in age groups with high seroprevalence (Nielsen et al., 2012; Nielsen, 2012).

Overall, faecal culture-positive prevalence was low, but generally higher the younger the age group. This corresponds well with previous studies on clinical expression of *S*. Dublin in cattle herds, where calves were more frequently and more severely affected by the disease than older cattle (Richardson and Watson, 1971; McDonough et al., 1999). One of the reasons for the varying prevalence observed in field studies is that *S*. Dublin is a very dynamic infection within cattle herds. Moreover, in some barn sections the cattle populations are also very dynamic with a continuous or fluctuating number of new animals with varying susceptibility to the infection being introduced to the age groups over time (Nielsen et al., 2007; Nielsen et al., 2012).

Furthermore, a seasonal trend was seen, in that the faecal culture-positive prevalence was generally highest in the winter season (i.e. December to February). This differs from the pattern observed for outbreaks of *S*. Dublin in cattle herds, which tend to have the highest incidence from August to November (Steffensen and Blom, 1999; Carrique-Mas et al., 2010). Again this may be explained by differences between outbreak and endemic situations which may be related to variations in infectious doses and immunity levels in different age groups, management, hygiene, herd size and other diseases in the herd (Wray and Sojka, 1981; Steinbach et al., 1996; Nielsen et al., 2012).

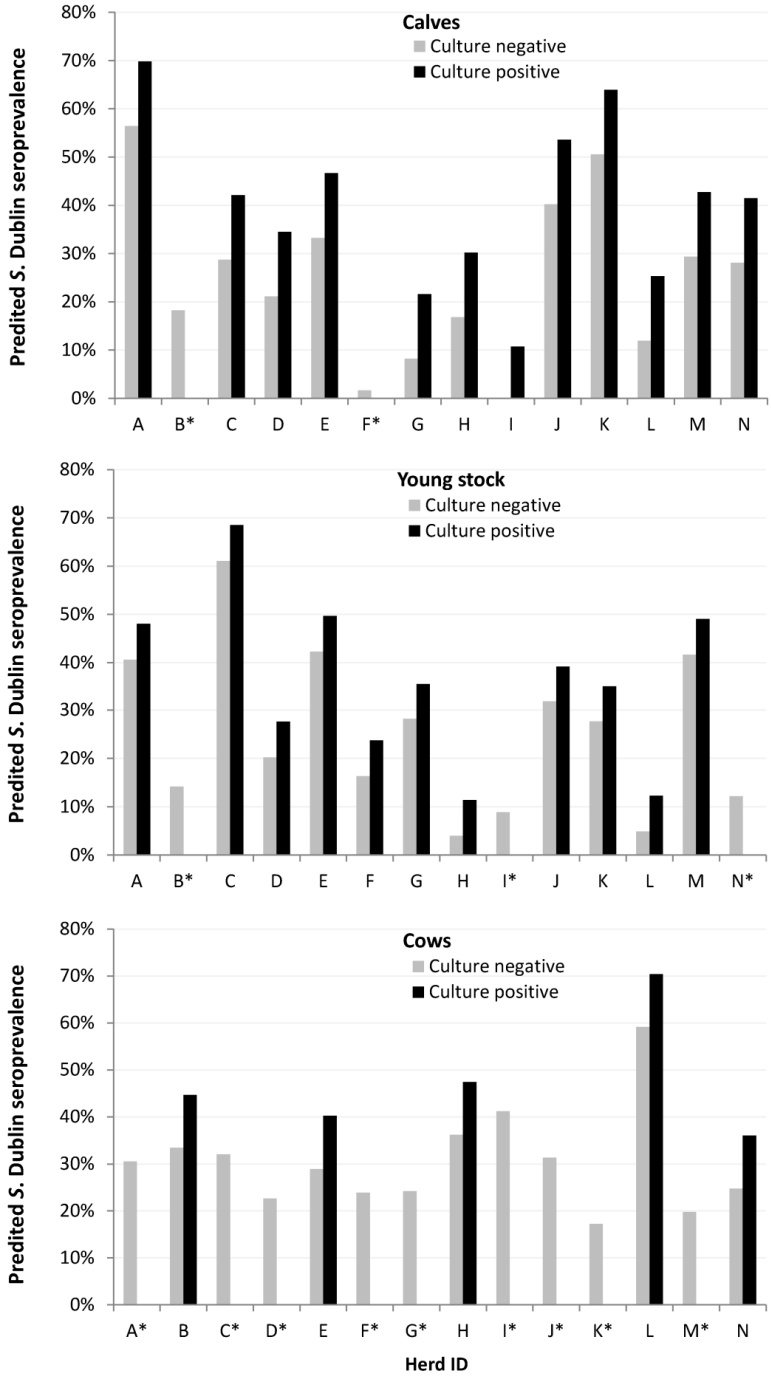

Figure 4 Model predicted seroprevalences in three age groups in 14 *S*. Dublin infected dairy herds. The grey bars represent seroprevalence in the situation where there was at least one positive faecal culture sample, the black bars represent the situation where there were no positive faecal culture samples in the age group (*= no positive faecal cultures in that herd- and age group).

This is the first study providing this level of longitudinal and extensive data collection for investigation of within-herd *S*. Dublin epidemiology. All cattle present in the barns were sampled at each visit (i.e. animals on pastures were excluded from the sampling rounds) leading to a total of 10,162 paired samples for analysis of antibodies and bacteriology. Samples consisted of rectally collected faecal samples making it possible to follow the excretion patterns of each individual animal over time. However, the low diagnostic sensitivity of the faecal culture method is generally problematic in studies of *S*. Dublin infections (Nielsen et al., 2004). This may have affected the associations and correlations found between faecal culture prevalence and seroprevalence. It is likely that the associations and correlations would have been stronger if there had been more positive faecal culture samples. However, the seasonal and age difference would most likely have been the same, as it is unlikely that biased sensitivities and specificities of the laboratory tests were present in this study. A cut-off of 50 ODC% was used to differentiate between serologically negative and positive samples in the subsequent descriptive and statistical analyses. Sensitivity and specificity of the used laboratory tests depend on age as described in previous papers (Nielsen and Ersbøll, 2004; Nielsen et al., 2004). The sensitivity was very low for young calves. Therefore, the observations from calves below 90 days old were left out for the statistical analyses of correlations and associations. In Denmark, it is recommended not to use serology for calves below 3 months of age in order to avoid the sensitivity issues with the test.

In conclusion, this study provided detailed estimates and illustration of dynamics and factors affecting seroprevalence and faecal culture-positive prevalence of *S.* Dublin in all age groups of endemically infected Danish dairy herds. These results can be used in modelling of infection dynamics and control scenarios, as well as planning of test strategies to support surveillance and control programmes at herd and national levels.

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PAPER III

Salmonella Dublin infection in young dairy calves: Transmission parameters estimated from field data and an SIR-model

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Abstract

In this study we used field data collected from October 2001 to January 2002 to estimate number of days of faecal excretion of *Salmonella* Dublin bacteria and time to seroconversion in infected calves below the age of 180 days. Based on these estimates all calves in four endemically infected dairy herds were grouped into the following infection states: susceptible (*S*), infectious (*I*) and resistant/recovered (*R*). Resistant calves had either acquired maternal antibodies through colostrum or they have recovered from previous infection and had a high level of antibodies directed against *Salmonella* Dublin possibly protecting them from becoming infected again until the level of antibodies had decreased to sufficiently low levels. Using the antibody measurements and faecal excretion periods, it was possible to assign the most likely infection state to each calf per week of the study period.

Estimates of transmission parameter, β, were obtained from a generalised linear model relating the number of new infections to the proportion of susceptible and infectious calves per week. From β, the reproduction ratio *R* at steady state and the basic reproduction ratio R_0 were estimated for each herd and across herds. The R_0 denotes the average number of new infections caused by one infectious individual that is introduced to a fully susceptible population. The point estimates for R_0 ranged from 1.1 to 2.7 in the study herds. However, the confidence intervals were wide. Data were too limited to show possible significant differences in the parameters between the study herds. However, the tendency in the data suggested that there may be important differences. Across herds the R_0 was close to two suggesting that on average one infectious calf will produce two new infectious calves when introduced into a fully susceptible population under typical Danish dairy production systems. Further, the analyses indicated that environmental contamination from infectious calves plays an important role in transmitting *Salmonella* Dublin between calves.

Introduction

Salmonella Dublin is a cause of concern in the cattle industry, because it is a zoonosis causing severe invasive infections in humans and because it causes economic and welfare losses in infected herds (Peters, 1985; Helms et al., 2003). The infection has a tendency to become endemic in many cattle herds in Denmark. When attempting to control *Salmonella* Dublin infections in such dairy herds it is critical to intervene in the calf barn where the infection spreads readily. However, not much is known about the infection dynamics of *Salmonella* Dublin in calf barns of endemically infected herds, because most information comes from outbreak situations and clinical cases (Richardson and Watson, 1971; Wray et al., 1989). Knowledge about the basic reproduction number, R_0, is useful for modelling the infection and the effect of potential intervention strategies. The net reproduction number, *R*, at steady state is one, meaning that on average every individual that becomes infected succeeds in transmitting the infection to one other individual during its infectious period (Anderson and May, 1991, p. 17). However, R_0 must be above one for any endemically stable disease, meaning that when one infectious animal is introduced into a fully susceptible population on average more than one animal will become infected and thus outbreaks

may also occur. In endemically infected herds, the proportion of susceptible animals varies over time. Thus, the infection may die out, or a new outbreak may occur. The size of the outbreak is mainly related to the number of susceptible individuals in the herd (Anderson and May, 1991, pp. 68–69). This is supported by varying clinical signs over time and fluctuating seroprevalence of *Salmonella* Dublin in infected herds that makes it reasonable to assume that even in endemically infected herds, smaller outbreaks are occurring intermittently over time.

The aims of the study were to (1) estimate length of the infectious periods and serological response to infection in calves below 180 days of age from field data, (2) illustrate fluctuations in size of the infection states S (susceptible), I (infectious) and R (recovered/resistant) over time and (3) to estimate the transmission parameters, β, R and R_0 for *Salmonella* Dublin among young calves (<180 days old) in four Danish dairy herds with long-term infection on the premises.

Materials and methods

Study herds and sampling

The estimates were obtained by the use of field data collected in Denmark in 2001–2002 and a generalised linear model relating the number of new infections to the proportion of susceptible and infectious calves per week. The data was collected as part of a large project known as ‘‘the Kongeåproject’’ through which previous knowledge of the four study herds was gathered (Andersen et al., 2000). These four herds were included in the study because they had several *Salmonella* Dublin positive cultures over a period of at least 1 year. They were therefore considered endemically infected with the bacteria. Clinical signs of salmonellosis were not obvious in these herds before the study period began. The sample collection was organised so that all calves that were born in the study period (a total of 88 calves) were sampled every 3–4 days for the first 4 weeks after birth and then once per week. All neighbouring calves in the same barn areas were sampled once per week. In total 181 calves were sampled in the study period. The number of calves varied between 16 and 69 per herd. Calves were sampled between 1 and 24 times each, on average 9.4 (S.D. = 7.2) times. Every sample event involved collection of an un-stabilised blood sample from the jugular vein and a rectally collected faecal sample. It was attempted to collect a minimum of 25 g of faecal matter at each sampling. However, this often proved difficult in the very young calves. Blood samples were transported to the Veterinary Department of Steins Laboratory in Ladelund for detection of antibodies directed against *Salmonella* Dublin lipopolysaccharide (LPS) as described below.

Bacteriology

Faecal samples were cultured in the above-mentioned laboratory for presence of salmonella bacteria by a conventional method described and evaluated elsewhere (Nielsen et al., 2004). The sensitivity of the faecal culture method has been estimated to be between 6% and 32% depending on the age of the animal when pooling of samples was used before individual follow-up on positive pools. In the present study, all faecal samples were cultured individually and the calves were very young, so the sensitivity was close to the highest obtainable, probably around 25–50% (Richardson and Fawcett, 1973). The specificity was assumed to be 100%, as typing of all salmonella-positive isolates was performed at the Institute for Food and Veterinary Research in Copenhagen.

ELISA

Blood samples were analysed for presence of antibodies directed against *Salmonella* Dublin O-antigen based LPS using an enzyme-linked immunosorbent assay (ELISA) that has been described

in detail and evaluated elsewhere (Nielsen and Ersbøll, 2004; Nielsen et al., 2004). An ODC%-value, which is a background corrected ratio of the test sample optical density (OD) to a known positive reference sample, was calculated for each sample as follows:

$$ODC\% = \frac{\left(\overline{OD}_{sample} - \overline{OD}_{neg\ ref} \right)}{\left(\overline{OD}_{pos\ ref} - \overline{OD}_{neg\ ref} \right)} * 100\%$$

where $\overline{OD}_{sample}$ is the mean value of two test wells, $\overline{OD}_{neg\ ref}$ and $\overline{OD}_{pos\ ref}$ are the mean values of four reference wells in the ELISA plates, respectively. The sensitivity of the serum ELISA at the cut-off value used in the present study (25 ODC%) was approximately 40–46% and the specificity 89–98% for animals between 0 and 99 days of age (Nielsen and Ersbøll, 2004; Nielsen et al., 2004). For calves from 100 days and older, the sensitivity was estimated to be 82–85% and the specificity 88–97%. The reason for the low sensitivity in calves younger than 11–12 weeks of age is most likely due to a poor capability to produce antibodies by this age group of calves. This was documented in another study and had to be taken into account when determining the infection states of the calves in our study (Da Roden et al., 1992). The non-optimal specificity may be due to maternal antibodies in this age group. Seroconversion was defined as at least a doubling of the ODC% to above 30 between two sample events. These criteria were based on a mix of results from previous studies and practical experience with the ELISA (Robertsson et al., 1982; Nielsen, 2003).

Infection status of the calves

To analyse data for transmission parameters, the infection status susceptible (*S*), infectious (*I*) and recovered/resistant (*R*) of all calves was determined for every week of the sampling period by both faecal shedding and by serology. In the absence of reasonable sensitivity of the bacteriological culture method, serology offers another way to determine the infection status (Veling et al., 2000). Calves were given status *S* when there was no bacterial growth in the faecal samples and the ODC% was below 25. Status *I* was assigned from the day that calves had a positive bacteriological culture and 17 days onwards. This average period was estimated from the data from culture positive calves (see results). Additionally, calves were assigned status *I* based on seroconversion. The infectious period was set to start 36 days prior to the recorded date of seroconversion and 17 days onwards from that date, if the calf was below the age of 100 days at time of seroconversion.

Seroconversion in calves older than 100 days lead the infectious period to be estimated to begin 14 days prior to seroconversion and the infectious period would be set to be shorter (12 days). Status *I* was followed by status *R* for 14 days unless new infection occurred within those 14 days. In that case, the calf was defined to be continuously infectious. Status *R* was also assigned to calves that had an ODC% above 25 and were not culture positive. This could for example be newborn calves with maternally derived antibodies or calves that continued to have high antibody levels beyond the designated 14 days recovered period following an infectious period.

Because calves older than 1 month were sampled on a weekly basis, the time step for the analyses was a week. Therefore, calves that were sampled twice weekly were assigned the same status for the whole week. When calves became infectious (changed from *S* to *I*) and when they recovered (went from *I* to *R*), the whole week was assigned *I*. This aggregation of data into weekly steps changed the minimum infectious period from 12 or 17 days to 3 weeks in the model. When calves were losing their maternal immunity (went from *R* to *S*), the whole week was defined *S*. New

infections were defined each time a calf became infectious after a susceptible period. Examples of infection groups for two calves are shown in Fig. 1A and B.

Statistical analysis

To estimate the transmission parameter, β, we used the framework of a simple SIR model for transmission of *Salmonella* Dublin between calves. The model is illustrated in Figure 2. Homogeneous mixing of the calves was assumed. Calves were considered born into either the *S* or the *R* compartment depending on whether they received *Salmonella*-specific antibodies through colostrum. After an infectious period, calves were considered resistant for at least 14 days or until their antibody levels fell below the cut-off value of 25 ODC%.

New infections were assumed to occur at the rate $\beta((SI/N) + E)$, where β is the infection rate, *S* the number of susceptible individuals, *I* the number of infectious individuals, *E* an external environmental infectious component and *N* is the total number of animals present in the given time period (Geenen et al., 2005). According to this model, the number of new infections, *C*, in each time interval, Δt, was assumed to be Poisson distributed and had the following expected value ($e(C)$):

$$e(C) = \beta\left(\frac{(SI)}{N} + E\right)\Delta t$$

log(β) was estimated with a generalised linear model (GLM) using the Genmod procedure in SAS®, Version 9.1 (SAS Institute Inc., 2002) with the response variable *C*, $\log(SI/N + E)\Delta t$ as offset (with Δt being 1 week) and a log link function. The external component (*E*) was added to correct for potential infection from the environment of the calves when no infectious calves were present. Because *E* was unknown, several levels of *E* from very low (0.001) to high (0.2) was tested in the model to check model fit to the data and to evaluate the effect of the size of *E* on the parameter estimates. To estimate the 95% confidence interval for log(β), the standard error (S.E.) was calculated as the two-sided confidence coefficient assuming a normal distribution and multiplied by the standard error from the model: log(β) ±1.96*S.E. The overdispersion parameter was estimated from the scaled deviance statistics (McCullagh and Nelder, 1989). The overdispersion parameter allows for possible dependence between grouped animals. Also, from a more practical point of view, it ensures that any lack-of-fit that remains after careful inspection and possible modification of the model, is reflected by larger standard errors and more conservative inference. An overdispersion parameter close to one indicates that the data follow a Poisson distribution.

The basic reproduction ratio (R_0) is the average number of secondary cases per week produced by one infected individual during the entire infectious period (Diekmann et al.,1990). R_0 was estimated by the following formula: $R_0 = \beta/\gamma$

where γ is the recovery rate and $1/\gamma$ is the estimated average infectious period in weeks which was estimated from the field data.

Another approximate method used to estimate the R_0 for *Salmonella* Dublin in the study herds was used to check the influence of the external component (*E*) on the above estimates. This method assumes that at equilibrium an approximation of the R_0 is related to the proportion of susceptible individuals in the population: $R_0 = 1/(S/N)$. The proportion of susceptible individuals was calculated as the average proportion of susceptible individuals over the entire study period.

The net reproduction number *R* was calculated as R_0 multiplied with the proportion of susceptible calves (Anderson and May, 1991, p. 17). This method would only apply if the *E*-component was low.

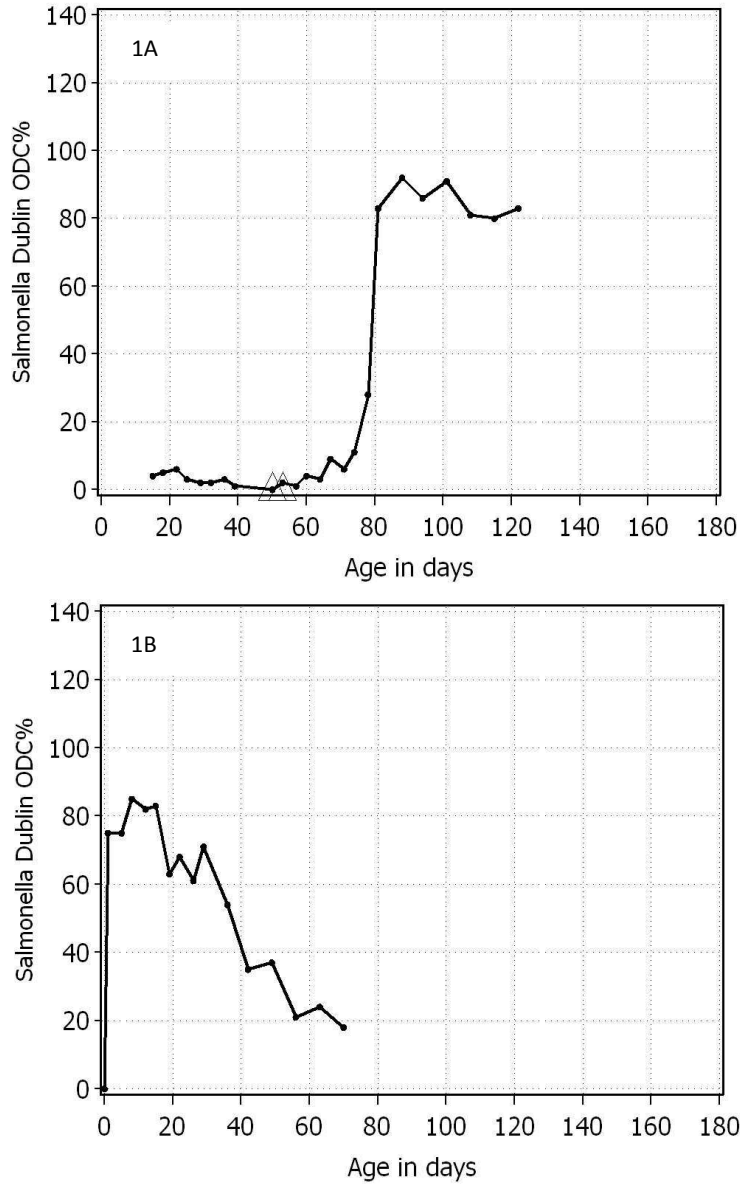


Figure 1 Antibody measurements (ELISA) (lines), faecal shedding (Δ=positive for *Salmonella* Dublin) and infection status (*S*, *I* or *R*) in two calves. The calf in 1A was defined *S* from day 17 to day 52 of age, then I until 71 days of age and finally *R* from 71 to 121 days of age at which point it left the calf barn. The calf in figure 1B was defined *R* from birth to 55 days of age and then *S* for the remainder of the sampling of that calf. The first sample was precolostral and therefore at 0 ODC%. After uptake of maternal antibodies the ODC% rose to very high levels.

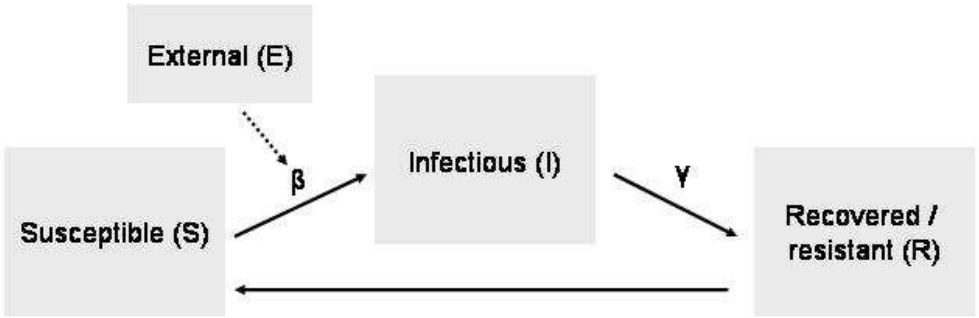


Figure 2 Compartments and pathways in the SIR-model used for estimation of the *Salmonella* Dublin transmission parameter, β, in four endemically infected dairy herds. *E* is an external component that allows for new infections to occur due to environmental contamination and γ is the recovery rate.

Results

Time of infectiousness and seroconversion

Based on the laboratory results of 19 calves that shed Salmonella Dublin in the study period, the average time of infectiousness (shedding of bacteria) was estimated to be 17 days (range 3–68 days) and the average time from onset of shedding to seroconversion in calves in this age group was estimated to be 36 days (range 11–67 days) (Table 1). For the model this resulted in a minimum infectious period of 3 weeks due to aggregation of data into weekly time steps. New cases appeared to arise in seven out of 16 (44%) weeks with no infectious animals in the previous week as opposed to 11 out of 48 (23%) weeks with infectious animals in the previous week.

Table 1 Descriptive statistics for 19 calves (*N*) that were faecal culture positive for *Salmonella* Dublin in four endemically infected Danish dairy herds.

Variables	N	Mean	Std.dev.	Median	Min-Max
Age at start of infectious period (in days)	19	40	23	43	3-70
Infectious period (in days)	19	17	19	10	3-68
Age at seroconversion (in days)*	10	75	15	76	52-100
Time from start of shedding to seroconversion*	10	36	17	28	11-67

*Nine animals that excreted bacteria did not show seroconversion in the study period.

Table 2 Estimated transmission parameter (β), standard error (SE) and basic reproduction number (R_0) for *Salmonella* Dublin in young calves in four Danish dairy herds based on an average infectious period of three weeks and the risk posed by environment contamination fixed at 0.1. Proportion of susceptible animals (*S*) and the net reproduction number (*R*) over the study period.

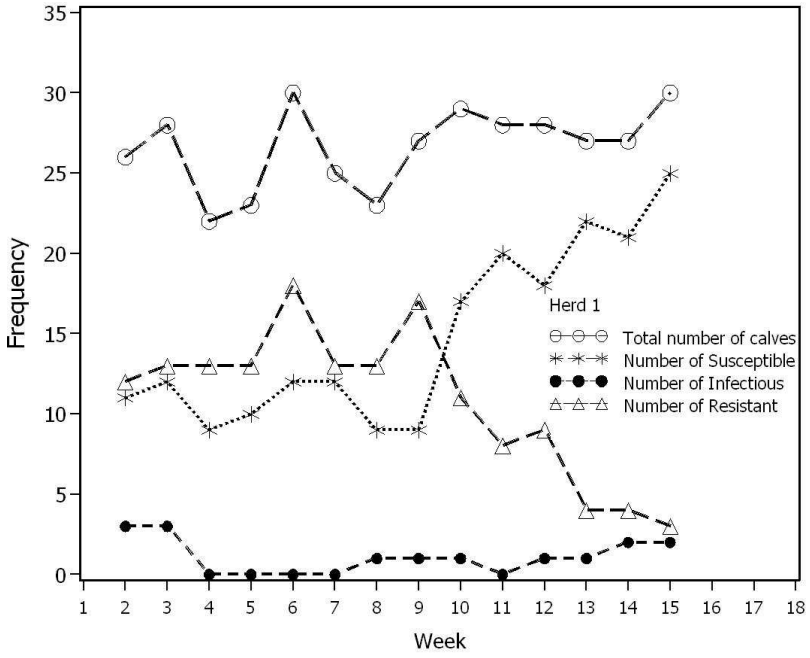
	Log(β)	SE	R_0	95% CI of R_0	Proportion *S*	*R*
Herd 1	-1.00	0.90	1.1	0.2 - 6.4	55%	0.6
Herd 2	-0.13	0.45	2.6	1.1 - 6.3	37%	0.9
Herd 3	-0.51	0.63	1.8	0.5 - 6.3	54%	0.9
Herd 4	-0.11	0.59	2.7	0.9 - 8.5	52%	1.3

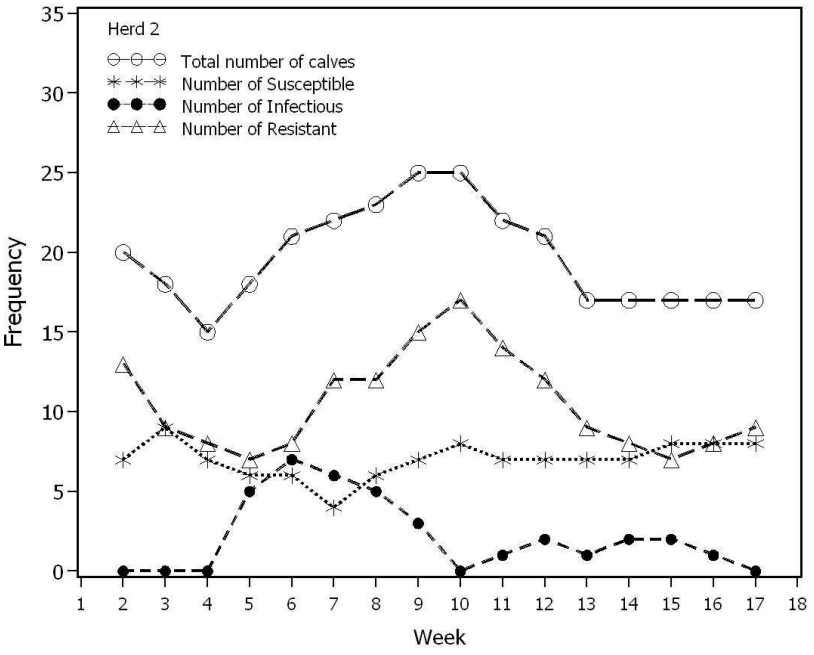
Transmission parameters

Table 2 contains the results of the log-linear regression for the four herds as fixed effects with *E* set to 0.1 which produced reasonable model fit, and Table 3 contains the estimate across all four herds with a correction for repeated observations within herd for different levels of *E*. The log-linear model with the four herds as fixed effects was overdispersed (deviance/d.f. = 2.4). The average infectious period in weeks that was used to estimate R_0 was 3 weeks. Though the estimates appeared to vary between the herds, the confidence limits were wide and therefore significant difference between herds was not demonstrated. Fig. 3a–d illustrates the fluctuations in the size of the different infection states *S, I, R* and total number of calves (*N*) per week of the study period according to the data and the definitions. Herd two had a peak in infections, which can be considered a small outbreak among the calves in weeks 5–8. This was reflected in the R_0 estimate for this herd.

Table 3 Estimated transmission parameter (β), standard error (SE) and basic reproduction number (R_0) and model fit evaluation (log likelihood) for *Salmonella* Dublin in young calves across four Danish dairy herds based on an average infectious period of three weeks at different levels of risk posed by environmental contamination as opposed to transmission by direct contact (*E*).

E	Log(β)	SE	R_0	95% CI of R_0	Log likelihood
0.001	-0.21	0.13	2.4	1.9 - 3.1	-27.4
0.01	-0.21	0.13	2.4	1.9 - 3.2	-27.9
0.1	-0.31	0.14	2.2	1.7 - 2.9	-28.7
0.2	-0.44	0.15	1.9	1.4 - 2.6	-29.1





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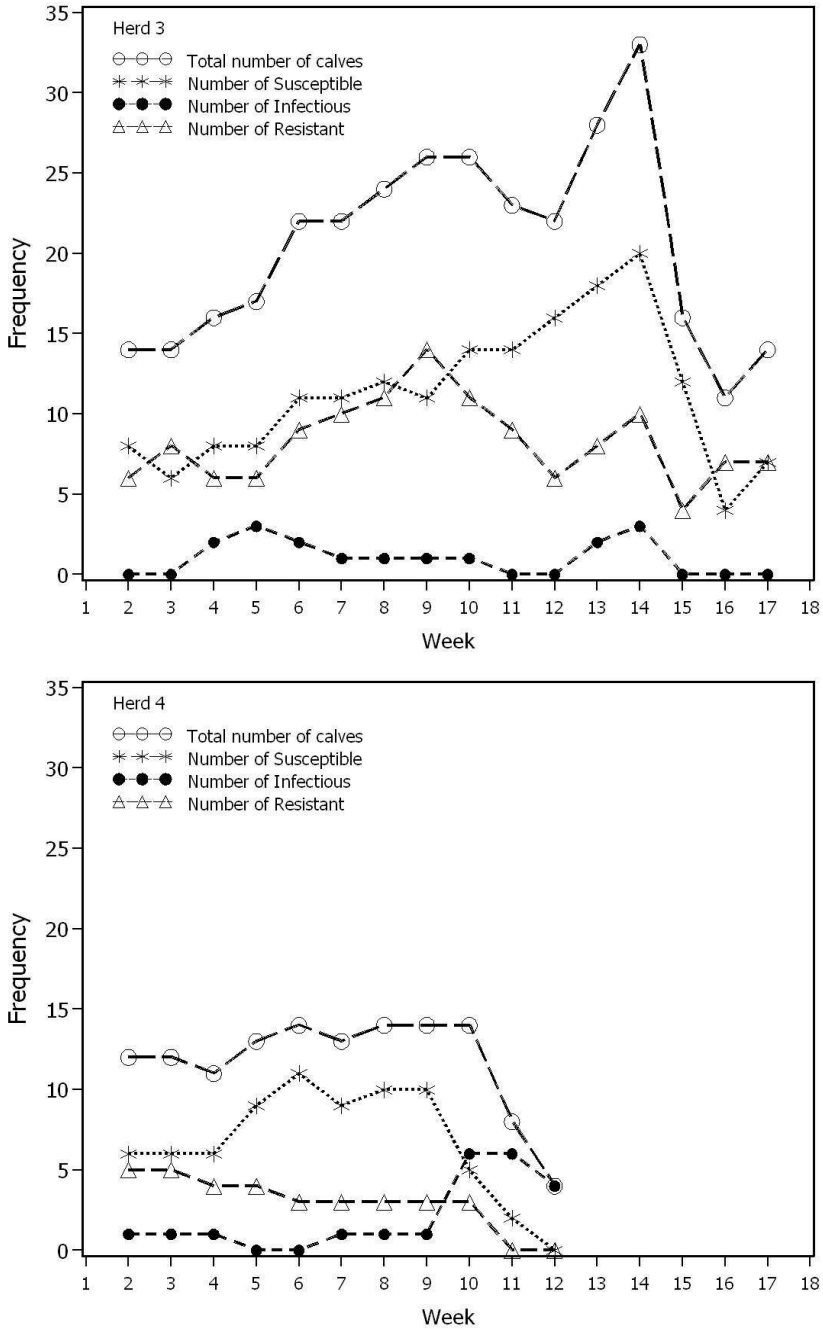

Figure 3 The dynamics of the size of infection groups *S, I, R* and the total number of calves, *N*, in the population per week of the study period in four dairy herds. The large fluctuation in *N* is due to bull calves being sold from the herds around two weeks of age and movement of calves in groups between barn areas.

Herd four appeared to have experienced a similar outbreak in weeks 10–12, but the herd was very small and thus there were only few observations available for the model estimations resulting in a

very wide confidence interval. Across herds, the R_0 estimate of *Salmonella* Dublin was significantly higher than one for all tested values of *E*, indicating that upon introduction to a fully susceptible calf population an infectious calf would on average infect approximately two other calves and therefore be likely to cause an outbreak. The estimates from the approximate method of R_0 calculation were similar (R_0 = 1.8–2.7) (Table 4) to the R0 estimates from the model (R_0 = 1.1–2.7) (Tables 2 and 3).

Table 4 Approximation of the basic reproduction number (R_0) for *Salmonella* Dublin in young calves based on the average proportion of susceptible (*S/N*) individuals in a 12-16 study period in four Danish dairy herds.

Variables	*S/N*	Std. Dev.	*N*	R_0	95% CI of R_0
Herd 1	0.55	0.18	14	1.8	1.6 - 2.2
Herd 2	0.37	0.09	16	2.7	2.4 - 3.1
Herd 3	0.54	0.11	16	1.9	1.7 - 2.1
Herd 4	0.52	0.24	11	1.9	1.5 - 2.6
Across herds	0.49	0.17	57	2.0	1.9 - 2.2

Discussion

The data set was unique in that all young calves in four herds were sampled at least once a week for 12 weeks. The data were used to estimate the transmission rate of *Salmonella* Dublin based on the new cases in each time period in young calves. However, the data only covered calves up to 180 days of age. It would be preferable to be able to include several age groups or the entire herd. Such data collection is extremely time-consuming and expensive, in particular if bacteriological culture needs to be performed on all samples. Because ELISA measurements do not give a very good indication of whether an animal is infectious, recovering from infection or a latent carrier, bacteriological culture is needed for this type of study (House et al., 1993; Hoorfar et al., 1996; Veling et al., 2000). On the other hand, it is known that conventional bacteriological culture also lacks sensitivity in cattle faecal samples and there is large variation in the duration of excretion of bacteria between individual calves (as illustrated by our data in Table 1). Correct classification into infection states is therefore difficult to obtain for this infection (Richardson and Fawcett, 1973; Nielsen et al., 2004). Thus, we may have underestimated both the number of infectious calves in each time step and the number of new infections, which again could affect the R_0 estimates.

For optimal estimation of transmission parameters, the time between each sampling should preferably be as short as the average generation interval, i.e. the time from one animal becomes infectious to the time the second case infected by the first case becomes infectious. For *Salmonella* Dublin the generation interval is probably only between 3 and 7 days, which is why at least weekly samples are required if the estimations are based on field data. Infection rates, duration of infection and recovery rates could possibly be estimated using a Bayesian model though the data used here may be too limited to improve the posterior estimates. For the present model we have mainly used the serological changes over time and faecal culture results rather than single ELISA results. Therefore, adjusting for sensitivity and specificity is not easily done. In a Bayesian model knowledge about test accuracy could be included. The Poisson model is only an approximation to the real transmission dynamics. In particular, when the number of susceptible animals is small and the infection intensity high, then the expected number given by the model will overestimate the true expected number in the next time step. In this study this is unlikely to affect

the estimates, because the expected number of new cases was never high, and the number of susceptible animals rarely low (Fig.3).

The R_0 estimates around two indicated that *Salmonella* Dublin would not spread very rapidly through susceptible populations under management systems similar to the ones in these herds. This makes sense because Salmonella Dublin is an infection that primarily spreads via the faecal-to-oral route and under typical Danish dairy herd conditions young calves with individual housing up to about 6–8 weeks of age, do not necessarily have a lot of direct contact between many neighbouring calves. On the other hand, direct contact between calves may not be the only factor leading to transmission of infection. High contamination of the environment by infectious calves and to some extend adult cows may also lead to transmission. In a simulation model it would be possible to allow the contamination level of the environment to depend on the number of infectious animals in the barn area in the previous time steps.

The number of calves per herd was too small to determine the differences between herds but there was an indication that in some herds *Salmonella* Dublin may spread faster than in others, and that small outbreaks occurred during some time periods. This could be due to hygienic conditions in the herds, housing and management of the calves. Earlier studies on risk factors for the spread of Salmonella Dublin confirm that herd management, but also coinfections with other diseases such as BVD aggravate an outbreak (Wray and Roeder,
1987; Veling et al., 2002).

The point estimate for the reproduction ratio at equilibrium(R) was between to 0.6 and 1.3 which was expected, because the herds were infected with *Salmonella* Dublin for several years and thus were in an endemic situation. However, under endemic situations there may be fluctuations in the proportion of susceptible animals leading the net reproduction ratio to increase, meaning that the transmission of bacteria between animals has increased periodically, whereas during other periods the herd immunity level would be sufficiently high that no or very little transmission of bacteria would occur (Anderson and May, 1991).

The model fit to the data was not optimal. The model was overdispersed, which indicated that there was more variation in the number of new infections than expected and the standard errors of the transmission rate had to be inflated to correct for this effect. The poor fit was probably a result of the fact that at times there were no infectious calves in the herd but new cases did occur (Figure 3). Therefore, we included an external component (E) in the model, and the model fit did in fact improve with increased levels of environmental contamination indicating that this is an essential source of new infections. This suggests that E needs to be included in the model, however it is quite likely that the environmental contamination came from calves that where infectious not long before the weeks with no infectious animals present and that the bacteria survived in the environment. Thus, it is likely to be highly dependent on the number of infectious calves in the previous time steps. It is advisable to explore the effect of such an environmental component by a simulation model in which this component is allowed to vary stochastically or dependent on the number of infectious animals in the previous time steps. Another source of biased R_0 results is that we may have misclassified some calves as non-infectious though they were in fact shedding bacteria. Few studies were available to aid in defining the infectious periods and recovery rates, and the main study available was based on clinical experiments, but confirmed the time of infectiousness in our study (Robertsson, 1984). Also, it must be expected that the

individual variation of infectiousness is large. Such individual variation in length of infectious periods and time of onset of infectiousness was not fully included in the analyses.

The next steps will be to include the parameters in a stochastic simulation model, in which the heterogeneity among calves and the infectiousness of other age groups in the herd can be included. The external component could be an environmental compartment related to the number of infectious animals in the lactating cows and survival of the bacteria in the environment (Wray et al., 1989). However, this poses even higher demands on the data sources available.

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PAPER IV

Prevalence and risk factors for *Salmonella* in veal calves at Danish cattle abattoirs

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Prevalence and risk factors for *Salmonella* in veal calves at Danish cattle abattoirs

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Summary

The study's objectives were to determine herd- and animal-level prevalence and herd- level risk factors for *Salmonella* in dairy-bred veal calves at slaughter in Denmark. In total, 1296 faecal samples were collected at five cattle abattoirs in Denmark during 2007-2008. The animals came from 71 randomly selected specialised veal-calf producers that delivered more than 100 animals to slaughter per year. *Salmonella* Dublin bacteria were isolated from 19 samples from 12 herds and *Salmonella* Typhimurium was isolated from one sample. The apparent prevalence of herds delivering *Salmonella*-shedding animals to slaughter was 18% (95% CI: 9-27). The overall estimated true prevalence of shedding calves at slaughter was 1.3%. Veal-calf herds that purchased animals from herds not classified as low risk in the Danish *Salmonella* surveillance programme had significantly (*P*=0.03) higher risk of delivering *Salmonella*-shedding calves to slaughter. The results emphasize the importance of efforts in the dairy industry to ensure food safety for consumers.

Introduction

Salmonellosis is one of the most common zoonoses in the world. It was the second most commonly reported human zoonosis in the European Union (EU) in 2008 (Anonymous, 2007) with a total of 131,468 confirmed cases. The most common sources of human *Salmonella* infections were eggs, pork and poultry, and *Salmonella* (*S.*) Enteritidis and *S*. Typhimurium were the most frequently reported serovars. However, beef cannot be completely ignored as a source of *Salmonella* infection in humans even though most member states of EU, including Denmark, reported very low (< 1.0 %) proportions of beef carcass swab samples testing positive, although some member states reported higher prevalences (up to 7.5 %). In Denmark, approximately 16-36 human cases of salmonellosis were attributed to beef produced in Denmark and about the half number (3-25) to imported beef in 2008. Furthermore, for a large proportion (~40-55 %) of salmonellosis cases in humans the source was unknown (Anonymous, 2009). Some of these could be from beef or direct contact to cattle. In 2008, 28 people were recorded as being hospitalized in Denmark with *S*. Dublin. This serotype is known to be more invasive, difficult to treat and leads to higher mortality in humans than other *Salmonella* serotypes (Helms et al., 2003). This particular serotype is host-adapted to cattle. Thus the source of human cases of *S*. Dublin is likely to be either beef produced in Denmark, imported beef or direct contact to infected animals and their surroundings.

Specialised veal-producers purchase bull calves from dairy herds around the age of 2 weeks and rear these animals until ready for slaughter. In Denmark, most veal calves are slaughtered before the age of 12 months. They may be infected with *Salmonella* if bacteria are present in the rearing herd or they may become infected or contaminated during transportation and lairage at the abattoir. In a study from 93 abattoirs in UK in 2003, seven different *Salmonella* spp. were found in 36 / 2,553 (1.4 %) faecal samples collected from cattle slaughtered at age <30 months. *S.* Mbandaka was found in 10 (27.8 %), *S.* Typhimurium in 10 (27.8 %), *S.* Dublin in eight (22. 2%) and *S.* Derby in four (11.1%) of the 36 positive samples. The median age of the tested cattle was 24

months (Milnes, 2008). An older study of apparently healthy veal calves in UK found 31/ 720 (4.3 %) animals tested infected with *Salmonella*. Twenty-three of these were *S.* Dublin. However, only eight (1.1 %) of the tested animals had *Salmonella* in the intestinal contents. The rest were found infected by culture of internal organs, lymph nodes or carcass surfaces (Nazer and Osborne, 1976). In a review of the importance of *S.* Dublin in humans in Denmark Lester et al. (1995) concluded that reduction in human *S.* Dublin infections could be obtained through stricter regulations for the slaughtering of animals from *S.* Dublin-infected herds, optimal hygiene at abattoirs and increased cooperation between the veterinary and medical professions concerning investigation of routes of infection. It is therefore relevant for the veterinary authorities and the Danish beef industry to learn more about prevalence, serotype distribution and risk factors for *Salmonella* infection in cattle at slaughter.

There is a lack of studies of risk factors for *Salmonella* in veal calves. However, suggestions of factors of importance for *Salmonella* occurrence in cattle generally include hygienic factors in the herds e.g. flies in pens (Vanselow et al., 2007), contact with poultry manure or wild bird manure, outdoor calving, herd size and herd expansions (Warnick et al., 2001). Hygiene and contacts at markets and in vehicles are also likely to be important risk factors before slaughter (Wray et al., 1991). In Danish dairy herds, risk factors for becoming infected in 2003 included herd size, number of purchased cattle from test-positive herds and number of test-positive neighbour herds. Organic herds were less likely to recover than conventional herds indicating that different types of management can influence the occurrence of *Salmonella* in cattle herds (Nielsen et al., 2007). Off-farm rearing of heifers has been acknowledged as an important risk of infection with multi-drug-resistant *Salmonella* in US dairy herds (Adhikari et al., 2009). One study also reported that for heifers and cows, recent antimicrobial treatment increased the probability of isolating *Salmonella* from faecal samples (Warnick et al., 2003). Also, *Salmonella* has been associated with high calf mortality in dairy herds. This may be due to both direct effects of the infection and underlying management factors (Nielsen et al., 2010).

The Danish Cattle Federation and the Danish Veterinary and Food Administration initiated an active surveillance programme for *S*. Dublin in 2002. In short, the program consists of testing based on regular bulk-tank milk testing of dairy herds and blood sampling mainly at slaughter of non-dairy herds (Warnick et al., 2006). All collected samples are tested for antibodies directed against serogroup-D *Salmonella* antigens. *S.* Dublin is by far the most important serogroup-D *Salmonella* type for cattle. Whereas herd classification in the programme is not perfect, it is aimed at classifying herds into groups of low (level 1) and high (level 2) risk of being infected with *S*. Dublin infection, plus a third group (level 3) with herds diagnosed with clinical salmonellosis from *S.* Dublin. Level 1 herds that purchase animals from level 2 herds become classified as level 2 herds for a period of at least 3 weeks and until new tests from the herd allow it to be promoted to level 1 (Jordan et al., 2008). This has markedly reduced the movement of animals from level 2 herds to level 1 herds and has contributed to a marked reduction from 26 % to 9 % national dairy herd-level prevalence from 2002 to 2010. The incidence of human cases of *S.* Dublin has not decreased proportionally over the same time period. Trade and hygienic slaughter restrictions apply to level 3 herds. However, it is not clear to what extent cattle from level 1 and 2 pose a risk of introducing *Salmonella* to the abattoir and whether trade and hygienic slaughter restrictions may be relevant for other herds than those with clinical salmonellosis in order to further improve food safety.

The objectives of this study were 1) to estimate the prevalence of specialised veal production herds that deliver *Salmonella* to abattoirs in Denmark via shedding animals, 2) estimate the prevalence of veal bull calves that carry *Salmonella* bacteria in the colorectal contents at slaughter and determine the concentration of bacteria in *Salmonella*-positive faecal samples and 3) to determine herd risk factors for *Salmonella* in veal calves at slaughter. Such risk factors can potentially be used to classify high-risk herds so that special hygienic measures can be taken at transportation, lairage and slaughter to avoid contamination of carcasses.

Materials and methods

Selection of herds

In the study design the aim was to sample 20 bull calves from each of approximately 80 specialised veal-calf herds from 1 October 2007 until sufficient samples had been collected by sampling a maximum of five animals per delivery from each of these herds. Four cattle abattoirs from three different companies were selected by convenience, and they slaughtered calves from all over the country. Herds were selected based on number of slaughtered bull calves at these four abattoirs in the period 1 September 2006 to 1 September 2007. In total, 200 herds slaughtered more than 100 bull calves aged between 6 and 14 months at these four abattoirs in that period. Out of these 200 herds, 81 herds were randomly selected to participate in the project and sampling was initiated in November 2007. When sampling was stopped in April 2008, 70 herds had calves sampled at the four selected abattoirs and one herd was sampled at a fifth small private abattoir. The last 10 selected herds had either ceased production or changed to delivering calves to an abattoir not included in the study.

Collection of faecal samples

When bull calves between the age of 6 and 14 months from any of the selected herds entered the abattoirs on Mondays to Thursdays, the first five calves in the delivery were marked for sampling. If there were fewer than five animals delivered for slaughter in one day they were all sampled. After removal of the gastrointestinal tract at the slaughter line, faecal samples were collected by cutting into the rectum or colon with a hot-water sterilized knife. About 70 g faecal material was collected aseptically and placed in a container which was marked and stored at 4°C. Samples were sent to the analysing laboratory on the same day or the following morning.

Bacteriological culture method

Faecal samples were all examined at the Regional Northern Laboratory of the Veterinary and Food Administration (Aalborg, Denmark) according to ISO 6579:2002/Amd 1 2007 (International Organization for Standardization, 2007.) Faecal material (25 g) was mixed in 225 ml buffered peptone water (BPW) and left for pre-enrichment at 37°C for 18 ± 2 h. Inoculation of 0.1 ml test material onto modified semi-solid Rappaport-Vassiliadis medium base (MSRV agar) plates was followed by incubation for 48 ± 6 h at 41.5°C. MSRV plates were read after 24 ± 3h and after 48 ± 6 h. Material from MSRV plates suspected to be positive was inoculated on xylose lysine deoxycholate agar (XLD) (Oxoid CM0469), *Salmonella* chromogenic agar (SCA) (Oxoid CM1007) and modified Brilliant-green Phenol-red lactose sucrose agar (BPLS -agar) (Oxoid CM0329) plates and incubated at 37°C for 18 - 24 h. Isolates suspected to be *Salmonella* positive at XLD, SCA and BPLS were identified using *Salmonella* antiserum Poly A-I + Vi and API: ID 32 E. Serotyping and confirmation of positive isolates were conducted at the National Food Institute, Technical University of Denmark (Copenhagen).

All *Salmonella*-positive faecal samples were cultured by a semi-quantitative method, where the samples prior to pre-enrichment were diluted 10-fold with BPW. Five dilutions of each sample were examined separately as described above.

Data for risk factor analyses

The following explanatory factors were assessed in the statistical analyses: purchase patterns, herd size and calf mortality. Data for the risk factor assessments were collected from the Danish Cattle Database. All data were compiled at herd level. Purchase patterns were evaluated as the total number of animals purchased, number of animals purchased from herds not classified as low-risk herds and number of purchase events from herds not classified as low risk during the period 1 September 2006 to 1 September 2007 (the year before selection of herds for the study). Herd size was defined as the average number of male cattle in the herd during the same period. This number was adjusted for the number of days each animal spent in the herd.

The number of calves that died or were euthanized between ages 1 and 180 days from January 2007 to January 2008, adjusted for the average number of calves in the herds, was used as an estimate of the calf mortality at herd level. The calculation method has been described in detail elsewhere (Nielsen et al., 2010). The mortality percentage was transformed for the statistical analyses using the natural logarithm because the distribution was very right skewed.

Statistical analysis

Apparent herd-level and animal-level prevalence of faecal shedding was calculated directly from the laboratory results and an estimated true animal-level prevalence was calculated by adjusting the apparent prevalence estimates by the estimated sensitivity and specificity of the bacteriological culture test from a previous study (Baggesen et al, 2007).

The association between the herd probability of delivering animals shedding *Salmonella* to the abattoir (yes/no) and the explanatory factors described above was tested by logistic analysis using PROC GENMOD in SAS® v. 9.2 (SAS Institute, USA). All explanatory factors were included in a full model and removed by stepwise backwards elimination requiring a significance level of 5 % to remain in the final model. After reducing the model to only significant effects, all explanatory factors were then re-introduced one by one in the model to evaluate possible confounding and test for interactions with the main effects. However, total number of animals purchased, number of animals purchased and number of purchase events from herds not classified as low risk were highly correlated and could therefore not be included in the model simultaneously.

Results

Bacteriological culture results and estimated prevalences

In total, 1296 faecal samples were collected from 71 herds. Due to practical constraints at the abattoirs the number of samples collected from each herd was not easy to control and thus varied from 5 to 40 (mean =18.3, S.D. =6.5) in the final dataset. In total, 20 faecal samples were culture positive for *Salmonella* spp. The animals testing positive came from 13/71 herds. One herd had four positive samples, one herd had three, two herds had two and nine herds had one culture positive sample. All isolates were *S*. Dublin except one which was *S*. Typhimurium DT40. No herd had more than a single serotype isolated. It was possible to perform semi-quantitative estimation of *Salmonella* concentrations in 18 of the *S*. Dublin-positive samples. Sixteen samples had < 1

colony-forming unit (c.f.u.)/g faeces, one had between 1 and 10 c.f.u./g and one had between 100 and 1000 c.f.u./g.

The apparent prevalence of veal herds delivering animals carrying *Salmonella* infections in the colon or rectum to the abattoirs was 18.3 % (95% CI: 9.3-27.3). The overall apparent prevalence of culture-positive individual animals across all 71 herds was 1.5 % (95% CI: 0.9-2.2). In the 12 *S.* Dublin culture-positive herds the apparent prevalence of *Salmonella*-shedding animals varied from 4.3 % to 20 %.

The estimated true prevalence of shedding animals at slaughter was calculated assuming an average test sensitivity of 80 % based on a previous study of spiked samples with low concentrations of *S*. Dublin bacteria (Baggesen et al., 2007), and a test specificity of 99.5 % allowing for a small risk of cross-contamination of the samples at the abattoir or laboratory. The true prevalence estimate of shedding animals at slaughter was 1.3 % across all herds, and it varied between 4.8 % and 24.5 % in the 12 culture-positive herds.

Descriptive analysis of risk factors

Table 1 provides the distribution of risk factors for each *Salmonella*-positive and -negative herd.

Table 1 Distribution of risk factors with continuous outcomes (described by 25% (Q1) and 75% (Q3) quartiles) relative to bacteriological *Salmonella* status of 71 specialised veal herds.

Risk factor	Positive	Negative
Calf mortality		
Q1	0.034	0.025
Median	0.046	0.044
Q3	0.060	0.092
Herd size		
Q1	332	238
Median	521	357
Q3	878	579
Number of purchased animals		
Q1	301	309
Median	595	483
Q3	1227	844
Number of purchase events from herds		
Q1	4	1
Median	13	4
Q3	18	10
Number of calves purchased from herds		
Q1	73	12
Median	259	105
Q3	399	226

Risk factor analyses

One explanatory factor was significantly associated with faecal culture positivity in the logistic analysis, namely the number of times the veal herds purchased animals from herds not classified as low risk in the surveillance programme (intercept = -2.14, parameter estimate = 0.068, *P* = 0.03). The predicted association is depicted in Figure 1. None of the other confounders or

interactions was significant. However, number of animals purchased from herds not classified as low risk was borderline significant (*P* =0.06) in a univariable logistic regression model. Herd size was also borderline significant when included in a univariable logistic regression (*P* =0.06).

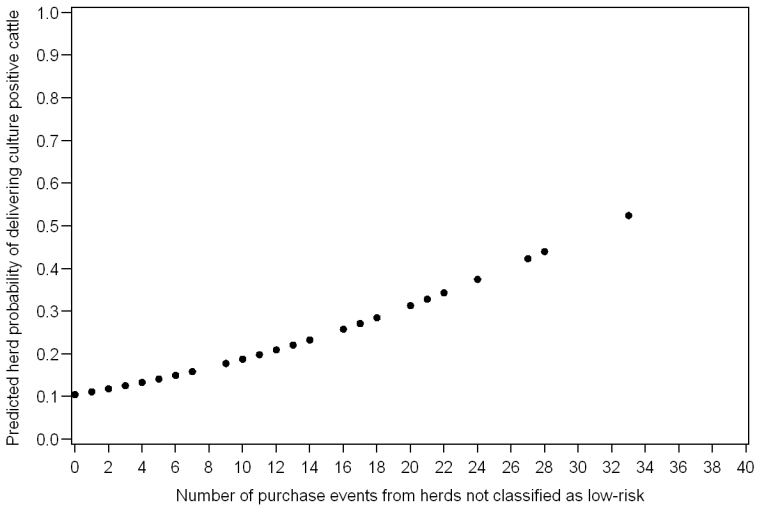

Fig. 1 Predicted probability of veal herds delivering *Salmonella*-shedding animals to slaughter vs. number of purchase events from high-risk herds during a one year period prior to sampling.

Discussion

In this study we estimated the prevalence and concentrations of *Salmonella* in faecal samples at slaughter in Danish dairy calves reared in specialized beef production herds that delivered more than 100 calves to slaughter per year. These herds are interesting because they contribute the highest load of *Salmonella* to the abattoirs due to the highest herd prevalence and the high number of animals delivered to slaughter at an early age. We found that at least 18 % of the veal herds delivered *Salmonella*-shedding calves to slaughter in the study period from November 2007 to April 2008. Some herds were probably misclassified as negative due to poor sample size and lack of diagnostic sensitivity of the bacteriological faecal culture test. The concentrations of bacteria in the culture positive samples were very low which further adds to lack of sensitivity in the culture test. We used a sensitivity of 80 % based on a previous study on spiked samples (Baggesen et al., 2007), but the concentrations may in fact have been lower in this study and other strains may have been involved, so we may have overestimated the sensitivity and thus underestimated the true prevalence of infected animals.

The herds were randomly selected amongst veal herds delivering more than 100 animals to slaughter per year, and sampling was attempted to be spread out over several deliveries, in order to estimate the true prevalence of animals carrying *Salmonella* bacteria in the intestines thereby acting as important reservoirs of contamination of carcasses at the abattoirs. The estimate of 1.3 % shedding animals corresponds well to the 1.4 % found in a similar study from UK (Milnes, 2008). However, the majority of the isolates were *S*. Dublin in our study, whereas in the study by Milnes et al. (2008) only 22 % of the isolates were *S*. Dublin. The overall prevalence might have been higher if we had collected the samples in late summer and early autumn instead of winter and early spring (Davison et al., 2005; Cummings et al., 2009).

Calf mortality was not associated with *Salmonella* shedding at slaughter in our study. This may be because there are other more common reasons for calf mortality in veal calves than *Salmonella,* including viral diseases such as BRSV and enzootic pneumonia.

Purchase of animals from herds that could not be classified as low risk (level 1) in the *Salmonella* surveillance programme was found associated with isolation of *Salmonella* from the herds. This is most likely because such herds are often infected with *Salmonella* and infectious animals are purchased into the veal herd. Similar associations have been found in dairy herds (van Schaik et al., 2002; Nielsen et al., 2007). This result is important because it provides an option for control of *Salmonella* in the cattle industry. If purchase from other than low-risk herds can be limited or stopped – for instance by reducing the number of infected dairy herds or by legislation against such trade, the number of infected veal herds can be reduced. This would be expected to lead to reduced input of infectious animals to the abattoirs.

Although the excretion of *S.* Dublin bacteria at the time of slaughter was generally low with an anticipated low impact on food safety, high shedders will occur at the slaughter line from time to time, but cannot easily be predicted based on register data and controlled *per se*. The results of this study strongly suggest that *Salmonella* infection can be controlled in veal herds by avoiding purchase of calves from infected herds. Most likely this should be supported by management aimed at controlling spread of *Salmonella* between calves within the herds.

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PAPER V

Bayesian estimation of true between-herd and within-herd prevalence of *Salmonella* in Danish veal calves

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Bayesian estimation of true between-herd and within-herd prevalence of *Salmonella* in Danish veal calves

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Abstract

Specialised veal producers that purchase and raise calves from several dairy herds are potentially at high risk of delivering *Salmonella*-infected animals to slaughter. However, the true prevalence of *Salmonella* infected veal producing herds and the prevalence of infected calves delivered to slaughter from infected herds are unknown in Denmark. Due to uncertainties about test sensitivity and specificity, these prevalences are not straightforward to assess. The objective of this study was to estimate the within-herd- and between-herd prevalence of *Salmonella* in veal calves delivered for slaughter to abattoirs in Denmark. Furthermore, it was investigated to which extent the estimates differed between a setup using both serological tests and faecal culture, compared to just serological tests, and whether the applied sampling scheme in the national surveillance programme in Denmark was sufficient to establish high posterior estimates of freedom from infection in individual herds. We used Bayesian analysis to avoid bias as a result of fixed test validity estimates. Serological test results from 753 animals and faecal culture from 1,233 animals from 68 randomly selected Danish veal producing herds that delivered more than 100 calves to slaughter per year were used to estimate the prevalences and estimates of freedom from *Salmonella*. Serological test results of 7,726 animals from 185 herds were used to compare the difference in prevalence estimates between serology alone vs. faecal culture combined with serology. We estimated that 34 to 57% of specialised veal producing herds were infected with *Salmonella*. Within the infected herds, 21 to 49% of the animals were infected. Few herds obtained high posterior estimates for the probability of freedom from infection given the collected data, with only six of 68 herds obtaining posterior probability of being infected less than 10%. Furthermore, this study indicated that serology is sufficiently sensitive and specific to be used for estimating the prevalence of *Salmonella*-infected specialised veal producing herds.

Introduction

Salmonella is a common food-borne pathogen with more than 3,600 human cases recorded in Denmark (Anonymous, 2009b) and more than 130,000 in the EU in 2008 (Anonymous, 2010). Less than 1% of these were attributed to beef, and few beef samples in the national fresh meat surveillance programme were *Salmonella* positive. However, 13 of the 16 *Salmonella* isolates in Danish beef and two of three isolates in imported beef were *Salmonella enterica* subsp. *enterica* serovar Dublin (*S*. Dublin) (Anonymous, 2009a). There are approximately 20-40 human cases of salmonellosis caused by *S.* Dublin infection every year in Denmark, and this type of *Salmonella* causes more severe infection and leads to higher mortality in humans than other serotypes (Helms et al., 2003). Hence, it is desirable to reduce the input of *S.* Dublin from cattle herds to cattle abattoirs.

More than 500,000 cattle are slaughtered every year in Denmark (Anonymous, 2009b). About half of these are young bulls or bull calves from specialised veal and beef production herds, where the bulls originate from dairy herds. There is an on-going surveillance and eradication programme for *S*. Dublin in Denmark (Anonymous, 2009b). However, the available diagnostic tests such as ELISA

and conventional bacteriological culture pose challenges for estimation of true animal- and herd-level prevalences, partly due to the uncertainty regarding the sensitivities and specificities of the available tests, which includes misclassification of the herds. Thus, currently the true prevalence of *Salmonella*-infected veal herds and the prevalence of infected bull calves delivered to slaughter from infected herds are unknown.

Only few studies have reported the apparent prevalence of *Salmonella* in veal calves delivered for slaughter and the lack of a test with high validity complicates estimation of true prevalence. Ranta et al. (2005) estimated true prevalence of *Salmonella* infected herds using bacteriological culture from lymph nodes at slaughter. They found between 0.54 and 1.36% of Finnish cattle herds infected. One Danish study found that overall, 1.3% of veal calves from specialised veal producers were shedding at slaughter (Nielsen et al., 2010). However, only bacteriological culture methods were used in that study, and even though the authors adjusted for the sensitivity and specificity of the test, they most likely underestimated true prevalence of infected animals. Warnick et al. (2006) suggested that at least five per cent of animals in *Salmonella*-infected dairy herds are likely to be test positive, when all animals are tested with both serology and bacteriological culturing. The maximum prevalence of test-positive animals within infected dairy herds reported in that study was 61%.

Bayesian methods have been used to obtain estimates of true prevalence of infections (Okura et al., 2010; Ranta et al., 2005; Branscum et al., 2004). Using prior information (specified as probability distributions) about test- and certain population-characteristics, it is possible to make posterior inferences about within-herd and between-herd prevalence as well as the posterior probability of individual farms being free from infection.

The objectives of this study were to estimate the within-herd- and between-herd prevalence of *Salmonella* in veal calves delivered for slaughter to abattoirs in Denmark. Furthermore, it was investigated to which extent the results differed between a setup using serological tests and faecal culture, compared to just serological tests and whether the applied sampling scheme in the national surveillance programme was sufficient to establish high posterior estimates of freedom from infection for individual herds.

Material and methods

Case definitions and herds

In this study, an animal was considered to be truly infected with *Salmonella* if it was a potential shedder of any serotype of *Salmonella* at slaughter. This covers different stages of infection in the individual animal including animals that are newly infected, persistently infected and infected in the gut without invasion of underlying tissues. Herds included in the study were herds predominantly producing veal calves. They were randomly selected out of all Danish herds delivering more than 100 bull calves to four selected abattoirs in the period September 2006 through August 2007 (n=200). The abattoirs were selected by convenience out of nine export-approved cattle abattoirs but they received calves from all over the country.

Sampling and test methods

Bacteriological culture

At each delivery of animals to the abattoir, samples were collected from the first five calves from the participating herds. If less than five calves were delivered at one time, they were all sampled.

The study aimed at collecting 20 samples from each herd, so a minimum of four sampling times were needed per herd. Seventy grams of faeces were collected via an incision in the rectum or colon after the digestive tract was removed from the carcass. Samples were transported to the Regional Northern Laboratory of the Veterinary and Food Administration in Aalborg for culture. . The culture method is described in detail in Nielsen et al. (2010), but briefly: 25 g of faeces were pre-enriched before 0.1 ml test material was inoculated onto Modified Semi-solid Rappaport Vassiliadis Medium Base (MSRV-agar) plates. Material from suspected positive MSRV-plates was inoculated on Xylose Lysine Deoxycholate agar, *Salmonella* Chromogenic agar and modified Brilliant-green Phenol-red Lactose Sucrose agar plates and suspect isolates were identified using *Salmonella* antiserum. Serotyping and confirmation of positive isolates and serotyping were performed at the National Food Institute, Technical University of Denmark, Copenhagen.

Serology

Blood samples for this study were collected as part of the Danish *Salmonella* surveillance scheme, where veal producing herds are classified based on serology in blood samples collected from animals delivered to slaughter (Anonymous, 2009b). In this scheme, one calf is sampled every month, but samples can be supplemented by the request of the owner. It is based on detecting immunoglobulin-G directed against serogroup-D O-antigens in serum by an enzyme-linked immunosorbent assay (ELISA) (Hoorfar et al., 1994; Smith et al., 1989). A sample was considered to be serologically positive if antibody level was above 50 ODC%, the cut-off level in the surveillance scheme. Animals with this level of antibodies are considered to be either currently or recently infected with *Salmonella*.

Datasets

Two datasets were extracted from the above sample and test schemes. Herds were sampled on multiple dates. Thus, the primary dataset contained all bacteriology results from each herd from November 2007 through April 2008 and all serology results from September 2006 through April 2008. These were used as a cross-sectional sample representing the infection status of the herd. Same procedure was applied to serology results for 2007 and 2008 for the secondary dataset.

The primary dataset – Serology and faecal culture

Due to practical and economical restraints, 80 herds were selected randomly from the herds delivering calves to the four selected abattoirs for the primary modelling. Out of these, 71 herds were tested by faecal and serological sampling in the study period. We only used results from 68 of the 71 herds because the last three were combined dairy and veal herds, which were classified according to bulk tank milk samples in the surveillance programme, and thus did not have blood samples collected routinely through the surveillance programme. Blood samples for serology were collected at the abattoirs from September 2006 through April 2008. Faecal samples for bacteriology were collected from November 2007 through April 2008. Thus, the two tests were not performed on the same animals. The sampling procedure and dataset are described in more detail in Nielsen et al. (2010).

The secondary dataset – Serology

This dataset included all herds from which more than 100 veal calves were delivered for slaughter yearly in 2007 and 2008 to the same four abattoirs as above. In total, 190 herds were registered in 2007 and 187 in 2008. Combined dairy and veal herds were excluded from the analysis as above,

which meant that in the final dataset for our study, 180 herds were included in 2007 and 162 were included in 2008. Across the two years 185 herds were included. Data used for the secondary dataset were serology results from the Danish surveillance programme in 2007, and combined serology results from the Danish surveillance programme and serology samples collected for another research project in 2008. Therefore, the number of samples per herd is higher in 2008. Samples were collected throughout the two years.

Statistical analysis

Model

A Bayesian model was constructed to estimate the prevalence of herds that delivered *Salmonella* infected calves to the abattoir as well as the prevalence of *Salmonella* infected veal calves in these herds. Since data was collected over a period of time it was not possible to determine the exact herd sizes. Furthermore, as herds delivered more than 100 veal calves for slaugther per year, we assumed that herds sizes were relatively large compared to the sample sizes. Thus, we chose to model using the binomial rather than the hypergeometric distribution (Hanson et al., 2003b). The model was adapted from Branscum et al. (2004). Briefly, the model assumes that for a herd, the following association between number of tested (n) and test positives (r) for serology (nsero,rsero) and bacteriology of faecal culture (nbact,rbact) and the true within-herd prevalence (ph) is implied:

$r_{sero}|p_h, Se_{sero}, Sp_{sero}, \sim Bin(p_hSe_{sero}+(1-p_h)(1-Sp_{sero}),n_{sero})$
$r_{bact}|p_h, Se_{bact}, Sp_{bact}, \sim Bin(p_hSe_b+(1-p_h)(1-Sp_{bact}),n_{bact})$
$p_h \sim Beta(\mu\sigma, \sigma(1-\mu))$ with probability p_{inf}
$p_h = 0$ with probability $1-p_{inf}$
$p_{inf} \sim Beta(a_{inf},b_{inf})$
$\mu \sim Beta(a_\mu,b_\mu)$
$\sigma \sim Gamma(s_\sigma,r_\sigma)$
$Se_{sero} \sim Beta(a_{Se\text{-}sero},b_{Se\text{-}sero})$
$Sp_{sero} \sim Beta(a_{Sp\text{-}sero},b_{Sp\text{-}sero})$
$Se_{bact} \sim Beta(a_{Se\text{-}bact},b_{Se\text{-}bact})$
$Sp_{bact} \sim Beta(a_{Sp\text{-}bact},b_{Sp\text{-}bact})$

where p_{inf} represents the probability that a herd is infected, p_h is the true prevalence within an infected herd (assumed to be the same for the serology and faecal culture), μ and σ are distributions describing the mean and variability of the prevalence within infected herds (assuming a random effects model for within-herd prevalence), Se and Sp are distributions representing the prior information about the serological test (sero) and bacteriology tests (bact).
The model for the secondary dataset is similar, except that the distributions regarding faecal culture are omitted.

Priors

Priors were obtained from the literature and by eliciting expert information (Table 1). For the priors modelled using Beta distributions, the following procedure was used: Median and either 95% (99%) upper or lower limit were obtained from literature with the exception of 95% lower limit for specificity of bacteriology. This was based on expert opinion of the second author, who

has 11 years' experience with *Salmonella* research in cattle. Using this information, the corresponding parameters were calculated using the 'beta.select'-function of the 'LearnBayes' package in R 2.9.2 (R Development Core Team, 2009).

Table 1 Beta-values for priors for sensitivity (Se) and specificity (Sp) for serology and bacteriology as well as herd prevalence and within-herd prevalence used in the model. Elicited prior information used to calculate priors in R and references for estimates are also shown.

Variable	Elicited prior information	Beta distribution-values	Reference
Herd prevalence (p_{inf})[1]	Mode=0.38	(14.90,24,43)	(Anonymous, 2009c)
	99% min=0.17		(Anonymous, 2009c)
Within-herd prevalence (μ)	Mode=0.30	(7.29,16.58)	(Warnick et al., 2004) Warnick et al., 2004)
	95% max=0.50	(4.25, 2.00)	
Variability within-herd prevalence (σ)	98% max=0.50[2,3]	(8.00,2.00)[4]	
	99% max=0.55[2,3]		
Se serology (Se_{sero})	Mode=0.70	(4.24,2.00)	(Nielsen and Ersbøll, 2005; Nielsen et al., 2004)
	95% max=0.95		(Nielsen and Ersbøll, 2005a) 2005)
Sp serology (Sp_{sero})	Mode=0.95	(537.63,28.62)	(Nielsen and Ersbøll, 2005; Nielsen et al., 2004)
	95% min=0.93		(Nielsen et al., 2004)
Se bacteriology (Se_{bact})	Mode=0.60	(3.89,2.70)	(Baggesen et al., 2007)
	95% max=0.90		(Baggesen et al., 2007a))
Sp bacteriology (Sp_{bact})	Mode=0.995	(227.04,1.46)	(Eriksson and Aspan, 2007)
	95% min=0.98[2]		

[1]Variable name in model, [2]Based on expert opinion, [3]90% certainty, [4]Gamma distribution

The prior for the measure of between herd variability of the within-herd prevalence (σ) was determined using the method described in Hanson et al. (2003a). Essentially, this method consists of the following steps: The prior for the mean within-herd prevalence (μ) was elicited as described above. From this distribution the mean of μ, i.e. μ*, was calculated as $\mu^* = a_\mu/(a_\mu+b_\mu)$. Then the expert was asked to express her prior belief about the 90%-tile of the within-herd prevalence, conditional on μ*. This prior belief was expressed as a median and 95% upper limit for the distribution of the 90%-tile. We then estimated the σ_m and σ_u associated with these prior beliefs about the median and 95%-tile of the 90%-tile of the Beta(μ*σ, σ(1-μ*))-distribution, where μ* is

known. Finally, the 2 estimates σ_m and σ_u were then used to fit a Gamma distribution reflecting the variability of σ. The R-code for these procedures is available from the authors upon request.

Sensitivity analyses

We analysed the primary dataset with different priors for sensitivity of bacteriology, between-herd prevalence and within-herd prevalence to estimate the influence of the individual priors on the results. Sensitivity of bacteriology was tested using median 0.5 and 0.70, both scenarios with 95% upper limit set to 0.8. Sensitivity of bacteriology was furthermore tested with non-informative priors, i.e. Beta(1, 1). Two different prior within-herd prevalences were examined, namely mode 0.05 and mode 0.50, with 95% upper limit of 0.3 and 0.9 respectively. Between-herd prevalence was increased to mode 0.50 with 95% lower limit kept at 0.17. Furthermore, the primary dataset was also analysed without faecal culture results to assess the importance of a second test. Finally, the primary dataset was analysed excluding one herd that had three positive faecal culture results, but no serology-positive results. There were indications in subsequent data from this herd that cross-contamination at transportation or at the abattoir could explain these odd laboratory results.

Model implementation

The Bayesian model was implemented in OpenBUGS version 3.0.8 rev 479, with the first 10,000 iterations discarded as burn-in to allow for convergence and the following 50,000 used for posterior inference. Convergence of the Markov Chain Monte Carlo (MCMC) chains was assessed by visual inspection of Gelman-Rubin diagnostic plots as well as time series plots of selected variables. Due to autocorrelation between iterations for between-herd prevalence, sensitivity for bacteriology and specificity for serology, thinning to every 50^{th} observation was performed. Sample size was evaluated by comparing the standard deviation and the MC-error.

The primary and secondary datasets were analysed separately. In the secondary dataset information from 2007 and 2008 were also analysed separately due to the differences in sampling frequency between the two years.

Results

Primary dataset

In total, 1233 faecal samples were collected from the 68 herds in the primary dataset. Between 5 and 40 samples were collected from each herd, with an average of 18 samples per herd. Nineteen (1.5%) samples from 12 herds were positive for *Salmonella.* One isolate was *S.* Typhimurium, the rest were *S.* Dublin.

Between 3 and 42 serology samples were collected from each herd (mean: 11 samples). In total 756 serology samples were collected and 118 (15.6%) from 38 herds were ELISA-positive. The distribution of serological and bacteriological positive herds is provided in Table 2. When herds where considered infected if at least one tested sample was positive, apparent prevalence of infected herds based on bacteriology was 17.6% (95% CI: 8.6-26.7%) and 55.9% based on serology (95% CI: 44.1-66.7%).

Table 2 Distribution of bacteriology vs. serology positive and negative veal herds in the primary dataset

	Bacteriology		
Serology	Positive	Negative	N herds
Positive	10	28	38
Negative	2	28	30
N herds	12	56	68

Posterior estimates for true prevalence of infected herds, within-herd prevalence as well as sensitivity and specificity for serology and bacteriology for the primary dataset can be seen in Table 3. The posterior distribution of the probability that the individual herds are infected with *Salmonella* is given in Figure 1.a. The prior between herd-distribution is added for comparison (dotted line), i.e. the belief that a randomly selected herd from the population is infected prior to sampling. The graph shows that most herds tend to obtain either fairly low or fairly high posterior probabilities of being infected with *Salmonella*. The lowest estimated posterior probability for a herd being infected was 7.3%, and only six herds had a posterior probability for being infected less than 10%.

Results from the sensitivity analyses where the posterior differed markedly are given in Table 3. Posterior prevalence of *Salmonella* infected herds increased when prior between-herd prevalence was increased from mode 0.38 to 0.50. When increasing prior sensitivity for bacteriology to mode 0.70, both between-herd and within-herd prevalence decreased, whereas sensitivity of bacteriology and serology increased. Increasing prior within-herd prevalence to mode 0.50 and making it almost non-informative (betadist: 2.1, 2.1) decreased posterior sensitivity of serology. Only minor changes in posterior distributions were found when using non-informative priors for sensitivity of bacteriology, decreasing bacteriology sensitivity to mode 0.5 or decreasing within-herd prevalence to mode 0.05 (results not shown). When excluding the bacteriology results, within-herd prevalence increased from 21-49% to 24-59%, while smaller changes were seen in the other estimates (data not shown). Very small changes were seen in posterior distribution when excluding the one herd with positive bacteriology but negative serology results (data not shown).

Table 3 Selected results from the sensitivity analyses compared to results from the primary dataset using priors from Table 1

	Results primary dataset	Changed prior		
		Herd prevalence	Sensitivity bacteriology	Within-herd prevalence
Herd prevalence	0.45 (0.34-0.57)[1]	0.56 (0.39-0.74)	0.37 (0.24-0.51)	0.44 (0.32-0.56)
Within herd prevalence	0.33 (0.21-0.49)	0.29 (0.18-0.47)	0.16 (0.09-0.25)	0.42 (0.22-0.80)
Se serology	0.69 (0.47-0.93)	0.69 (0.47-0-93)	0.90 (0.70-0.98)	0.57 (0.34-0.89)
Sp serology	0.95 (0.93-0.97)	0.95 (0.94-0.97)	0.92 (0.89-0.94)	0.95 (0.94-0.97)
Se bacteriology	0.09 (0.05-0.17)	0.09 (0.09-0.17)	0.61 (0.45-0.74)	0.07 (0.03-0.15)
Sp bacteriology	1.00 (0.99-1.00)	1.00 (0.99-1.00)	1.00 (0.99-1.00)	1.00 (0.99-1.00)

[1]median and 95% posterior credibility interval

Secondary dataset

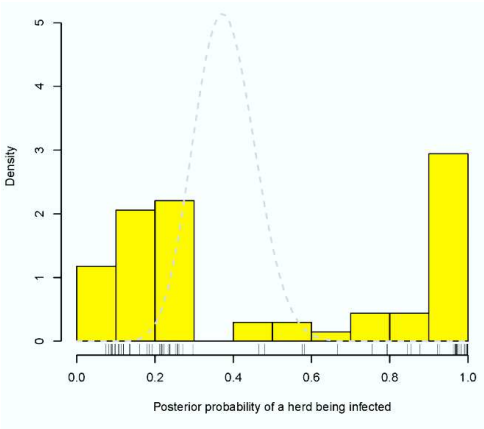
For the secondary dataset, the mean number of serology samples per herd collected in 2007 was 9 (min-max: 3-77), with a total of 1770 samples collected. For 2008, 5956 samples were collected with a mean of 37 (min-max: 3-786) per herd. The high number of samples in 2008 where due to a research project for which extra samples were collected in some of the herds, so 2007 represents routine surveillance data collection, whereas 2008 represents a scenario with increased sampling intensity. In Figure 1.b and 1.c, the posterior distribution of the probability of individual herds being infected is given with the prior between-herd prevalence shown as well for 2007 and 2008, respectively. For comparison, the posterior within- and between-herd prevalence for the secondary dataset is illustrated in Figure 2 along with priors and the posterior results from the analysis of the primary dataset and the primary dataset when excluding faecal culture results. Given the data for 2007, the estimate for within-herd prevalence was 26-53% and between-herd prevalence was 33-53%. These estimates are quite similar to those obtained with the primary dataset of 21-49% and 34-57% for within-herd and between-herd prevalences, respectively. The main result displayed in the figure is that the analyses including the new data suggested that the posterior prevalence estimates were higher than the prior beliefs about the prevalences of *Salmonella* in slaughter calves in Denmark.

Discussion

In this study we showed that analysing data using a methodology that takes into account prior knowledge about uncertain test validity estimates of ELISA and conventional bacteriological culture provides estimates of between-herd and within-herd true prevalence of *Salmonella* in specialised veal producing herds. In this situation where the validity of the tests is uncertain, this method is preferable to apparent prevalence values obtained in previous studies where the tests were either assumed to be perfect or true prevalence estimates were calculated based on fixed values of test sensitivity and specificity (Nielsen et al., 2010). The estimates obtained in our study are associated with large uncertainty (wide credibility intervals), but are based on the current knowledge as well as new data and probably represents the real situation that with the current knowledge and the additional data collected in this study there is still much uncertainty about the prevalence estimates.

Data for the primary dataset were collected over a period of 18 months. There was a maximum of four positive bacteriological tests from any herd so it is possible that it was only for a limited time that the herds included *Salmonella* shedding animals. Most herds contained more than a few positive serology samples and the antibody levels usually lasts longer after infection than shedding of bacteria (Nielsen et al., 2007). There may be a problem that herds considered positive by bacteriology in this study were not infected during the entire study period. However, since sensitivity of bacteriology is very low, shedding is intermittent and that *S.* Dublin can survive for long periods in the environment, we consider the classification of herds to be correct in most cases.

a)



b) ***c)***

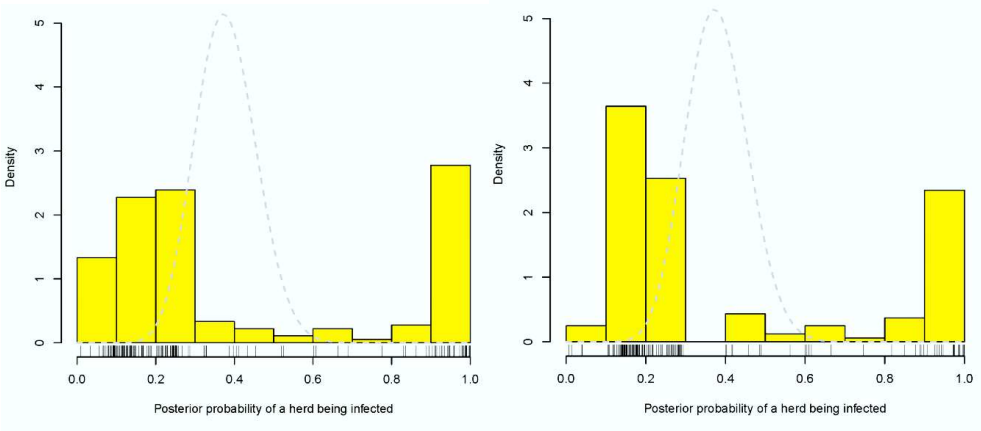


Figure 1 Histograms of the modelled posterior mean estimates of probability of *Salmonella*-infection for the individual herds (as well as rug plot of individual observations along the x-axis). The dotted line is the density function for the prior distribution based on literature, i.e. the prior belief that a randomly selected veal calf herd is *Salmonella* positive, Beta (14.90,24.43). a) is the estimates for the primary dataset with serology and faecal culture from 68 herds. For the secondary dataset, b) is the results from serology surveillance of 180 herds in 2007, and c) is the results from increased serological testing of 165 herds in 2008.

Herd selection

Herds were included in the study based on where they delivered calves for slaughter. Out of convenience four abattoirs were used for the data in the primary dataset, but these received animals from herds spread throughout Denmark. Herds included in the primary dataset were selected randomly from herds delivering to these four abattoirs. Animals from each herd were selected as representative samples at slaughter for both blood sampling and bacteriology. In the secondary dataset we had blood samples from all herds that delivered more than 100 animals to the four abattoirs, thus we consider our results representative for all the larger veal producing

herds in Denmark. Further investigations are needed to assess the prevalences in smaller veal producing herds.

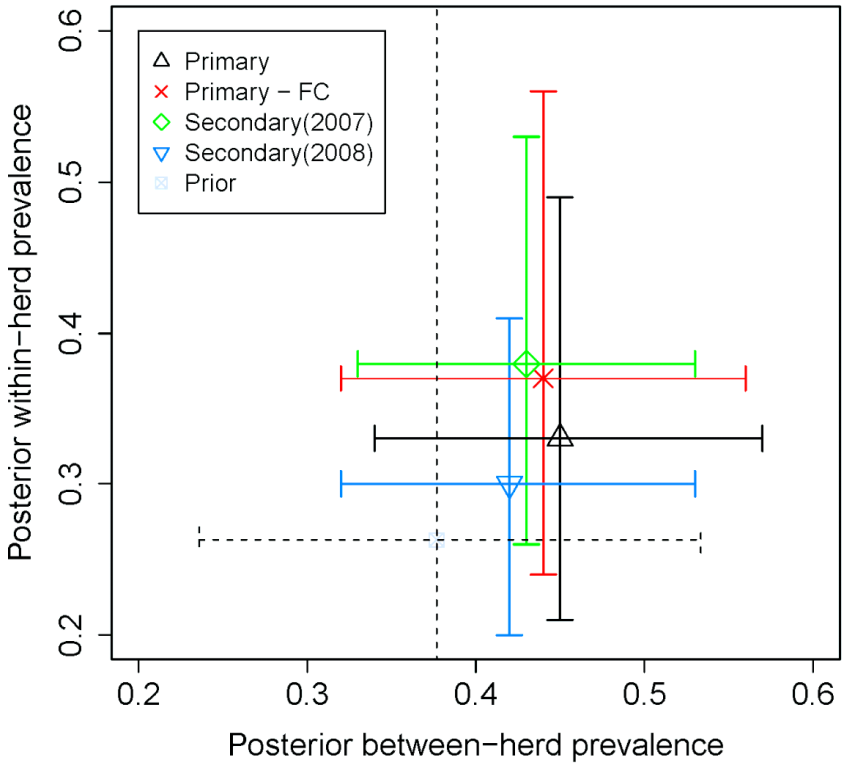


Figure 2 95% posterior credibility intervals for within-herd and between-herd prevalence of *Salmonella* for primary dataset with and without faecal culture (FC) as well as results for secondary dataset for 2007 and 2008. The secondary dataset from 2007 is representative of the national *Salmonella* surveillance programme in Denmark. Prior within-herd and between-herd prevalences are included as well (priors for within-herd prevalence ranging from 0.004 to 0.82).

Primary dataset

Estimated within-herd prevalences of *Salmonella* vary widely in the literature. Khaitsa et al. (2007) reported that the prevalence of *Salmonella*-shedding steers went from 0.7% to 62% in 143 steers over the first four months after being introduced to a feedlot. Other authors have reported prevalences from 0.08% to 46% faecal positive cattle at the abattoirs (Fegan et al., 2004; Barham et al., 2002; Donkersgoed et al., 1999). All those studies estimated apparent prevalence based on bacteriology, i.e. shedding animals and therefore underestimated the true prevalence of animals that can shed bacteria due to lack of sensitivity of bacteriology and intermittent shedding in infected animals (Baggesen et al., 2007; House et al., 1993). Our study estimates within-herd prevalence between 21 and 49% and reports on potential shedders, i.e. a combination of animals that are shedding at slaughter and animals with an immune response indicative of current or recent infection, which might or might not shed at the time of slaughter. Thus, our study might

overestimate the true within-herd prevalence of animals posing a risk of contamination at the abattoir to some extent.

We estimated that between 34 and 57% of veal producing herds were infected with *Salmonella.* Dargatz et al. (2003) found 37 of 73 and Fedorka Cray et al. (1998) found 38 of 100 feedlots positive for *Salmonella,* which is in accordance with our findings. Contrasting to this, Fegan et al. (2004) only found 9% of feedlots *Salmonella* positive. Only veal calf herds that delivered more than 100 calves per year for slaughter were included in our study, and smaller herds are less likely to be infected (Anonymous, 2009c). This could be due to fewer supplying herds, which means that there is less risk of introducing *Salmonella* to smaller veal calf herds (Nielsen et al., 2010).

Our model did seem to be able to determine if a herd was at high or low probability of being infected with *Salmonella* given the test results from the herd, and the primary dataset was divided into two groups with high and low probability of infection. Of the 68 herds, 18 herds had a probability of being infected greater than 0.95, while the lowest probability of a herd being infected was 0.07.Only six herds had a probability of less than 0.10. Hence, the model was unable to demonstrate (with 95% certainty) any herds free from *Salmonella* infection based on the available data. Five herds had a probability of being infected between 0.45 and 0.70 according to the model. These herds had either low prevalence of positive serology tests or had several positive serology tests but no positive bacteriology test results. This study aimed at collecting 20 faecal samples from each herd, but due to technical problems or too few animals delivered to slaughter during the study period, as little as five samples were collected from some herds which, in combination with few collected blood samples, were too few for the model to estimate if these herds were likely to be infected with *Salmonella* or not.

Transport to abattoirs has been reported to increase the apparent prevalence of *Salmonella* on hides (Barham et al., 2002), and from faeces from adult cattle but not feedlot cattle (Beach et al., 2002). It was not recorded for how long calves in our study were transported or kept in lairage before slaughter. In general, animals are transported relative short distances and are slaughtered the same day as they arrive at the abattoir in Denmark, which should reduce the risk of animals becoming infected after leaving the farm. However, cross-contamination or infection during transport cannot be ruled out from the results in this study, and it might be an explanation for the two herds with no positive serological tests but with positive faecal tests. The sensitivity analysis showed no effect of excluding one of these herds, so we assume that this did not affect results markedly.

Secondary dataset

Estimated within-herd prevalence was higher when based on serology results from 2007 than from 2008. This could be due to different test frequencies and sample sizes in the dataset between the two years, or it could be due to effects of simultaneous *Salmonella* eradication campaign in the Danish dairy herds, which the veal calves originated from. Lower between-herd or within-herd prevalence in 2008 than in 2007 in the dairy herds delivering calves to the veal calf herds could lower within-herd prevalence in the veal producing herds.

All estimated prevalences, based on the primary and secondary datasets, were slightly higher but narrower than the prior estimates. Estimates from the secondary dataset, where there were no bacteriology results included, were similar to estimates from the primary dataset. This indicates

that the more expensive bacteriology tests are not necessary when serology results are available for the herd. Estimates from 2007 as well as estimates from the primary dataset without bacteriology were slightly higher than estimates from the primary dataset including bacteriology, whereas estimates from 2008 were slightly lower. This could indicate that although bacteriology may not be necessary to estimate within- and between-herd prevalences, estimates based on the surveillance programme may overestimate the prevalences because of few samples collected per herd.

Choice of priors

We estimated a sensitivity of bacteriology between 5 and 17%. Other studies have reported sensitivities between 25 and 36% of bacteriology for calves tested at abattoirs, when culturing several organs and carcass samples from each animal (Nazer and Osborne, 1976; Watson et al., 1971). Both of those studies only used bacteriology results from organs or carcass to detect infected animals when estimating sensitivity of bacteriology of faecal samples and the sample sizes were small. Nielsen et al. (2004) included serology to estimate evaluate the true infection status of a large sample of animals. They found a sensitivity of 6-14% for bacteriological culture which is similar to what we found in this study. Sensitivity as high as 80-90% was reported by Baggesen et al. (2007), but that study was performed on spiked samples containing approximately 10 CFU/g faeces. In our study most of the positive samples only had 1 CFU/g (Nielsen et al., 2010), which could lead to lower sensitivity.

Most of the priors used in the model were found in the literature, but several estimates were based on studies from countries with different ways of producing veal than Denmark. However, similar results were found when using different priors suggesting that our data had a strong influence on the posterior estimates. Sensitivity analysis revealed that only when increasing prior estimate for the mode of sensitivity of bacteriology from 0.6 to 0.7 did results change markedly. This was not the case when decreasing sensitivity of bacteriology to 0.5 or when using non-informative priors. In view of the results and the literature, it seems more likely that sensitivity would be lower, rather than higher, than 0.6. Majority of estimates for the priors were based on literature regarding *S.* Dublin, and 13 of 14 isolates in the primary dataset were *S.* Dublin, thus we consider results from the model to be robust regarding the choice of priors.

Conclusion

This study shows that approximately 34-57% of specialised veal calf herds that delivered more than 100 calves to slaughter annually were infected with *Salmonella* in Denmark in 2007-2008. Between 21 and 49% of the animals within infected herds were potential shedders of *Salmonella* at slaughter. Furthermore, this study indicates that the surveillance programme in Denmark, based on relatively few serology samples per herd per year, is suitable to estimate the between-herd prevalence of specialised veal calf herds that might deliver *Salmonella* shedding animals to the abattoirs, but not able to demonstrate herds free from infection. The model presented here can calculate probabilities that individual herds are infected given the samples collected from the herd, which can potentially be used for herd classification in future control efforts.

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Conflict of interest

The authors declare they have no conflict of interest.

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PAPER VI

Risk factors for changing test classification in the Danish surveillance program for *Salmonella* in dairy herds

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Abstract

A surveillance program in which all cattle herds in Denmark are classified into *Salmonella* infection categories has been in place since 2002. Dairy herds were considered test negative and thus most likely free of infection if *Salmonella* antibody measurements were consistently low in bulk tank milk samples collected every 3 mo. Herds were considered test positive and thus most likely infected if the 4-quarter moving average bulk tank milk antibody concentration was high (≥25 ELISA ODC%) or if there was a large increase (>20 ELISA ODC%) in the most recent measurement compared with the average value from the previous 3 samples. The objective of this study was to evaluate risk factors for changing from test negative to positive, which was indicative of herds becoming infected from 1 quarter of the year to the next, and risk factors for changing from test positive to negative, which was indicative of herds recovering from infection between 2 consecutive quarters of the year. The *Salmonella* serotypes in question were *Salmonella* Dublin or other serotypes that cross-react with the *Salmonella* Dublin antigen in the ELISA, e.g. some *Salmonella* Typhimurium types. Two logistic regression models that accounted for repeated measurements at the herd level and controlled for herd size and regional effects were used. Data from 2003 was used for the analyses. A change from test negative to positive occurred in 2.0% of the quarterly observations (n=21007) from test negative dairy herds. A change from test positive to negative occurred in 10.0% of quarterly observations (n=6168) available from test positive dairy herds. The higher the numbers of test positive neighbor herds in the previous year-quarter the more likely herds were to become test positive for *Salmonella*. The number of purchased cattle from test positive herds was also associated with changing from test negative to positive. The bigger the herd, the more likely it was to change from negative to test positive. The effect of herd size on recovery was less clear. Large herds consisting mainly of large breeds or having test positive neighbors in a 2 km radius were less likely to change from test positive to negative, whereas the breed and neighbor factors were not found to be important for small herds. Organic production was associated with remaining test positive, but not with becoming test positive. The results emphasize the importance of external and internal bio-security measures to control *Salmonella* infections.

Introduction

Salmonella enterica subspecies *enterica* Dublin (***S.* Dublin**) is host adapted to cattle and causes health and economic losses in both the beef and dairy industries (Peters, 1985; Visser et al., 1997). Furthermore, it is a serious zoonosis and though human cases are rare in Denmark (between 26 to 43 cases yearly in 2001 to 2004) they are often fatal (Helms et al., 2003; Anon., 2004; Anon., 2005). *S.* Dublin is the most prevalent *Salmonella* serotype in cattle in Denmark, followed by *S.* Typhimurium. Therefore, it was decided to start a national surveillance program in Danish cattle with the primary goal of controlling *S.* Dublin. Data collection was started in 2001 and the program was launched in October 2002 by initiative of both the Danish Veterinary and Food Administration and the Danish Cattle Federation. In the surveillance program, all Danish cattle herds are classified into three infection categories (Anon., 2004). Dairy herds are classified based on bulk tank milk (**BTM**) samples that are tested using an ELISA based on a *S.* Dublin lipopolysaccharide antigen

(O:9,12) (Hoorfar et al., 1995). Other serotypes than *S.* Dublin may cross-react with this antigen because of O-antigens on the bacterial surface that are common to those carried by *S.* Dublin, e.g. O:12 in *S.* Typhimurium (Konrad et al., 1994; Hoorfar et al., 1995). Thus, the term "*Salmonella"* in this study implies *S.* Dublin or other serotypes that occur in cattle and can cross-react with the antigen used in the ELISA in the surveillance program. In Denmark, most of these would be *S.* Typhimurium (Anon., 2004).
For dairy herds, a *Salmonella* category is determined from the 4 most recent ELISA results. Samples are collected approximately every three mo, but extra samples at other intervals may also be requested by farmers. There has to be at least three wk and no more than 5 mo between each sample in order to be used for the *Salmonella* category determinations. The average proportion of background-corrected optical density value of the sample to a known positive control sample (**ODC**%) is calculated from these 4 measurements. Non-milk producing herds are classified based on ELISA measurements on blood samples that are collected routinely for evaluation of the herd status of bovine virus diarrhea or voluntarily submitted for analysis from animals above 8 mo of age (Nielsen et al., 2003). Category (**C**) 1 is considered most likely free of *Salmonella.* If enough blood samples have been collected and all are below the cut-off of 50 ODC% non-milk producing herds are classified C1. Dairy herds are classified C1 if the average ODC% of the last 4 BTM measurements is below 25 and no increase of more than 20 ODC% is found when comparing the last measurement to the average of the three previous measurements. Until March 2006, C2 and C3 were divided into two sub-levels. C2a was considered likely to be infected because of antibody responses above the cut-off values. C2b was not classifiable because of lack of data or because of contact to herds in C2a or C2b. Herds in C3a had clinical salmonellosis diagnosed by bacteriological culture (usually outbreak herds). C3b had the bacteria detected by culture, but clinical salmonellosis had not been diagnosed, or the herd had purchased cattle from a C3a or C3b herd. Laboratory results and classification categories are recorded in a database that is part of the Danish Cattle Database. The systematically collected data on *Salmonella* antibodies in all Danish dairy herds provides a unique opportunity to evaluate risk factors for the infection at herd level.

The use of BTM ELISA for herd classification of dairy herds was first suggested by (Hoorfar et al., 1994) who recommended the use of this test for screening and certification programs. In a small study (n=160 BTM samples) it was found that there was good association between herd history of salmonellosis, herd location and clinical status of the herd and the BTM ELISA response (Hoorfar et al., 1995). In the Danish surveillance program the BTM ELISA measurements of four samples are used for the classification, and the validity of the program testing scheme in dairy herds was evaluated on a large scale using a simulation model based on field study data from dairy herds known to be infected with *S.* Dublin and *S.* Typhimurium (Warnick et al., 2006). This study found good validity of the classification (sensitivity=95%, specificity=96%, negative predictive value=99% and positive predictive value=80%) at a national prevalence of 15% truly infected dairy herds. Infection was defined for that study as having at least one culture positive fecal sample from cattle or a within-herd prevalence of at least 5% based on individual animal ELISA results.

Risk factors for *Salmonella* infection in cattle at the herd level can be evaluated from two perspectives: 1) the risk of introduction of the infection to a previously uninfected herd; and 2) the risk of currently being infected with *Salmonella* bacteria. The introduction has been shown to be influenced by management practices of the herd and location of the herd. Trade of living animals, grazing with cattle from other farms and low level of bio-security for professional visitors have

been found as significant risk factors for introduction of infectious organisms to the herd (van Schaik et al., 2002). In a study of 1,429 Danish dairy herds the risk of becoming infected with *Salmonella* (measured as a change from negative to positive BTM ELISA response) increased when nearby neighbors were infected. Also, the risk increased with the prevalence of seropositive herds in the region (Wedderkopp et al., 2001). Pastures recently contaminated with infected slurry is also an important risk for new infection (Taylor and Burrows, 1971; Taylor, 1973).

The aim of our study was to identify factors influencing the risk of dairy herds changing test status as a measure of *Salmonella* infection or recovery. *Salmonella* serotypes of primary interest were *S*. Dublin, *S.* Typhimurium or other serotypes that may cross-react with the *S*. Dublin-antigen used in the BTM ELISA testing scheme in the Danish surveillance program.

Materials and methods

Data Sources

Data sources included the Central Husbandry Register, the Danish Cattle Database and data from the National Surveillance Program for *S.* Dublin. All live born cattle are ear tagged at birth and these three databases are more or less integrated and contain recordings of birth, location, movement, clinical records and laboratory results of all cattle in Denmark, thus providing data on herd size, breed, location, trading patterns etc. on all cattle herds. Geographical information on all farms was obtained from the Map and Land Register Authority in Denmark.

National Surveillance Program Data

For the objective of this study, we used the ELISA ODC% results from the National Surveillance Program database to recalculate *Salmonella* test results for all dairy herds. The test program validity and the relationship between BTM ELISA measurements and individual cow antibodies were described elsewhere (Nielsen and Ersbøll, 2005; Warnick et al., 2006). In another study, it was shown that mainly serogroup B-serotypes such as *S.* Typhimurium may cross-react with a *S.* Dublin antigen because of common lipopolysaccharide O-antigens 1 and 12 on the cell surface (Konrad et al., 1994). The herds were test negative if the four-BTM moving average was ODC%<25 and no increase of >20 ODC% was found when comparing the most recent measurement to the average of the three previous measurements. Test positive dairy herds included those with BTM ELISA results that exceeded either of the ELISA test cut-off criteria described above. Thus, all dairy herds were denoted either test positive or test negative for each sampling date. The actual regulatory categories used in the surveillance program (C1, C2a, C2b, C3a and C3b) were not used to define the response variables for these analyses, but C1 would be similar to test negative and C2a to test positive. Very few dairy herds were assigned C2b, C3a and C3b in the surveillance program and these herds were grouped according to their antibodies into either test negative or positive.

For the analyses, all herds had a *Salmonella* test result assigned to each year-quarter (**YQ**) based on the last 4 consecutive BTM samples. If a herd was classified more than once during the same YQ, because more than one BTM were collected, the test result for that YQ was selected randomly. The full dataset contained 70871 data lines from 8694 dairy herds from the period October 2001 to March 2004, and the herds had their *Salmonella* test result determined for between 1 and 11 YQ with a median of 9 and Q1 to Q3 of 8 to 10. Potential risk factors were constructed as either time dependent with measurements on a quarterly basis or as a one-time

recording representing the entire sampling period. For several of the variables, however, data were only available for a limited period as described in the section "Data Editing and Descriptive Statistics".

Non-milk producing herds were grouped based on blood samples collected either in the herds or at slaughter. The surveillance program in non-milk producing herds had changed several times in the period 2002-2004 and was most likely of varying accuracy compared with the program for dairy herds. Therefore, data from these herds were only used to construct variables concerning characteristics of neighbor. They were not included as study herds as such. Overall, the apparent prevalence of *Salmonella* infection in non-milk producing cattle herds was around 1.5%.

Study Herds

All dairy herds were included in the study regardless of whether they ceased operations during the period from which data was extracted (2001 to 2004). BTM measurements were available starting in 2001 when data collection was initiated. Dairy herds were defined as herds that had weekly somatic cell counts measured from BTM as part of a compulsory milk quality control program as this definition gives the most updated information on which herds were truly milk-producing herds. Out of the 8,694 dairy herds with adequate samples, 1,007 (11.6%) were defined as Jersey herds and 7,682 (88.4%) were defined as large breed herds (Holstein Friesian, Red Danish Cattle and mixed breeds) (5 herds had missing values for breed). The distinction between Jersey and large breeds was based on the geometric average percentage of fat in milk from weekly recordings in the Danish Cattle Database.

Data Editing and Descriptive Statistics

The following variables were extracted and constructed from the databases and used in the risk factor analyses. Even though some data (e.g. the *Salmonella* data) were available for the full period from 2001 to 2004, the final data sets used for the risk factor study had to be restricted to the four quarters of 2003 because of lack of data outside this period for most of the risk factors.

Table 1. Distribution of antibody test positive and negative dairy herds in each quarter of the year in the study period 2001-2004 in the surveillance program for *Salmonella* in Denmark.

Year and quarter[1]	Number of test positive dairy herds	Number test negative dairy herds	Overall apparent prevalence
2001, 4th	2016	5644	26.3%
2002, 1st	1962	6456	23.3%
2002, 2nd	1813	6173	22.7%
2002, 3rd	1841	5954	23.6%
2002, 4th	1752	5661	23.6%
2003, 1st	1684	5895	22.2%
2003, 2nd	1574	5714	21.6%
2003, 3rd	1449	5116	22.1%
2003, 4th	1555	5630	21.6%

[1] Data from 1st, 2nd and 3rd quarter of 2001 and 1st quarter of 2004 are not shown here because data was incomplete

Year-quarter. YQ were based on mo January to March, April to June, July to September and October to December for each yr 2001 to 2004. The distribution of test positive herds and test negative herds for each YQ in the study period is shown in Table 1.

Region. Geographical regions were constructed by dividing Denmark into 6 regions so that each of the six had approximately the same number of dairy herds and the area within the same region was geographically contiguous (except for separation by bodies of water) and had approximately the same apparent prevalence. In order to meet the latter criterion, North Jutland was further divided into two regions because of the large difference in apparent prevalence between the northern and southern part. The resulting 7 regions and apparent prevalence of each region in June 2003 are illustrated in Figure 1.

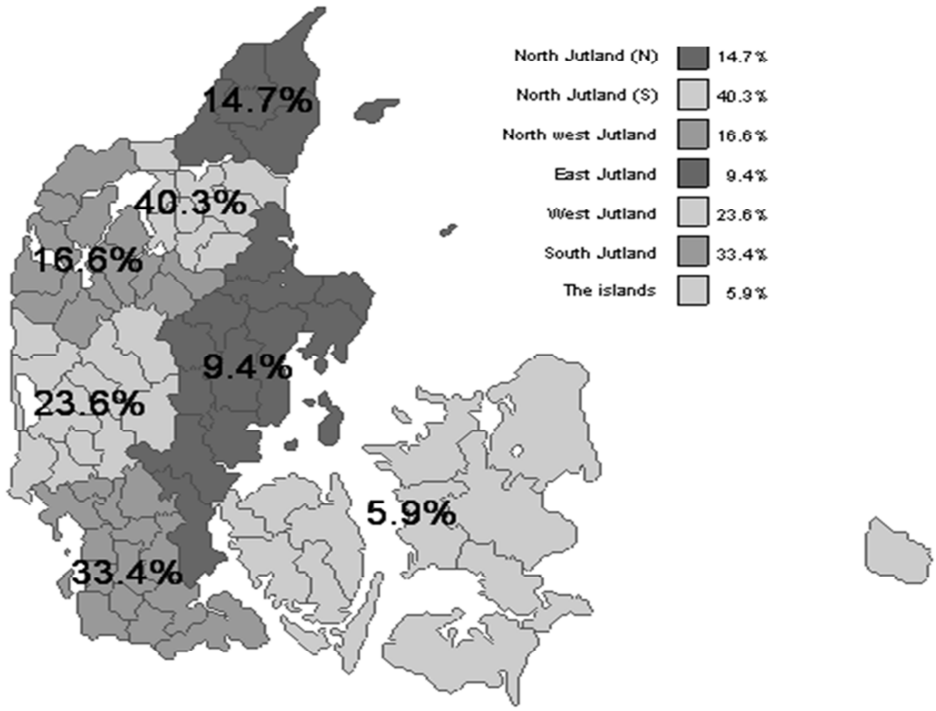

Figure 1 The 7 regions of Denmark and apparent prevalence of each region in June 2003 are illustrated.

Season. Season was defined as Winter (January to March), Spring (April to June), Summer (July to September) and Fall (October to December).

Organic. Eight percent of the 8,694 dairy herds were recorded as organic in 2003. Information about which herds were organic was obtained from the Danish Plant Directorate and merged with data from the Danish Cattle Database. Although organic status was coded as a time-dependent variable, for practical purposes it can be considered as non-varying because virtually all herds (>99%) were classified either as organic or not organic for the entire study period.

Neighbors in 2 km radius. The number of neighbors included beef and dairy herds in a 2 km radius around each study herd. This number was calculated for all of 2003, so that if a herd was recorded active at any point in time during 2003 it would be counted as a neighbor and would be assigned

neighbors as well. The number was used to represent the full study period as it was assumed not to change over time.

C2a or C3a neighbors. The number of cattle herds (beef and dairy) classified C2a or C3a in the surveillance program in a 2 km radius around each dairy herd in the previous YQ. Data were available from 1st YQ of 2003 to 1st YQ of 2004.

Close-contact neighbors. The legally-required area used to spread manure from each dairy and beef herd according to the regulations was calculated and the radii were calculated for circles equivalent to this area centered around each herd. The area depended mainly on the herd size and breed. This variable showed how many neighboring dairy and beef herd circles in which each dairy herd was located during the previous YQ. Data were available from 1st YQ of 2003 to 1st YQ of 2004.

C2a or C3a close-contact neighbor. Similar to the previous variable, except this variable only counted the number of circles from herds that were classified in either C2a or C3a in the surveillance program in the previous YQ.

Individual animal numbers, dates of birth, dates of entry to the holding, identification of the farm of origin, animal date and reason for departure, e.g. breeding, slaughter, export or death were extracted from the Danish Cattle Database. These data were used to construct the following variables:

Calf mortality. Calf mortality in the previous YQ was calculated as the proportion of live born calves that were ear-tagged and died within 1 to 90 d after birth. Data were available from 4th YQ of 2002 to 3rd YQ of 2003.

Source herds. Total number of dairy and beef source herds for cattle purchased in the previous YQ. Data were available from 2nd YQof 2002 to 1st YQof 2004.

Test positive source herds. Total number of test positive dairy source herds for cattle purchased in the previous YQ. Data were available from 2nd YQ of 2002 to 1st YQ of 2004.

Purchased cattle. Total number of dairy and beef cattle purchased in the previous YQ. Data were available from 2nd YQ of 2002 to 1st YQ of 2004.

Purchased cattle from test positive herds. Number of cattle purchased from test positive dairy herds in the previous YQ. Data were available from 2nd YQ of 2002 to 1st YQ of 2004.

Herd size. Total number of cattle (regardless of age and production type) on the premises counted per mo and averaged for each YQ. Data were available from 2nd YQ of 2001 to 4th YQ of 2003.

Statistical Method of Analysis

Two separate multivariable logistic regression models were used to analyze risk factors associated with a change of status between two YQ from test negative to positive and from test positive to negative, respectively. The factors analyzed in the model related to the YQ just before the change in test status (e.g. herd size, purchase of cattle, number of infected neighbors) or they could be factors that were the considered fixed over the full study period (e.g. number of neighbor cattle herds in a 2 km radius). Continuous variables were checked visually for a linear relationship with the log odds of the response variable before inclusion in the model. Because all variables other

than herd size did not show linear relationships it was decided to categorize these (see Table 2 for details on the categories).

The variables were assessed for co-linearity with the conclusion that total number of purchased cattle and number of source herds in the previous YQ could not be included in the models simultaneously. Neither could the number of purchased cattle from test positive herds and number of test positive source herds, number of neighbor circles and total number of neighbors in a 2 km radius, number of C2a or C3a-close contact neighbors and number of C2a or C3a-neighbors in a 2 km radius. The variables that were left out initially because of co-linearity were checked in the final model by replacing the analogous correlated variables.

The models were constructed as multivariable logistic regression models controlling for the correlation of repeated measurements from the same herd by using Generalized Estimating Equations (**GEE**s). All main effects were included in the initial model together with all interactions with herd size. Backwards selection was used to remove non-significant interaction terms and non-significant main effects in that order. Then 2-way interaction terms of the significant main effects were tested in the model. Significance level criteria for variables and interaction terms to remain in the final model were $P < 0.05$. For the variable selection all variables remained in the class statement in order to analyze on the same dataset. After the final model with significant effects was determined, the model was tested on a dataset including all data available for the class variables remaining in the model. This data set contained data from four YQ (1st to 4th YQ of 2003). This restricted data set was a result of lack of data for several risk factors as illustrated in the section on Data Editing and Descriptive Statistics. Odds ratios with 95% confidence limits were calculated as described by Hosmer and Lemeshow (2000). For the calculation of odds ratios for herd size and variables with significant interactions with herd size in the test positive to negative model three examples were selected for illustration (100, 200 and 300 cattle). It was necessary to choose specific numbers for the herd size as examples to illustrate the difference between combinations of risk factors, because herd size was a continuous variable in the models. In Denmark, a total herd size of 100 would be considered a small dairy herd, 200 a medium sized dairy herd and 300 a large dairy herd.

Software

SAS® version 8.2 was used for data editing and statistical analyses. The GENMOD procedure was used for logistic regression models with a repeated statement and an unstructured working correlation structure to account for the intra-herd correlation of response measurements within the same herd. The "withinsubject option" was used to allow for adjustment of correlation in the case of missing values for certain YQ.

Table 2. Descriptive statistics of categorical risk factors and changes in test results. Percentage (%) of dairy herds changing from test negative to positive or from test positive to negative out of the total number of herds in each scenario (N) according to antibody measurement data from the national surveillance program for *Salmonella* in Denmark.

		Previously test negative herds		Previously test positive herds	
Variables	Levels	N	% test negative to positive	N	% test positive to negative
Number of neighbors in a 2 km radius	0 - 5	5,379	2.3	1,098	15.7
	6 - 10	15,790	2.4	4,549	11.7
	11 - 15	16,553	2.6	5,418	10.9
	> 15	8,421	3.4	2,947	11.7
Number of neighbors classified as C2a[1] or C3a[2] in a 2 km radius in previous year-quarter	0	13,729	1.3	2,382	12.8
	1	5,214	2.2	1,608	11.3
	2	2,149	2.7	1,032	9.4
	> 2	1,996	4.1	1,694	7.7
Calf mortality in previous year-quarter (proportion)	0	12,502	2.1	2,875	13.4
	0 - 0.05	2,041	2.7	843	8.4
	0.05 - 0.2	5,274	3.2	2,065	9.5
	> 0.2	1,080	4.6	435	10.8
Number of cattle purchased in previous year-quarter	0	29,579	2.4	7,976	11.3
	1 – 10	8,572	3.1	2,777	12.2
	11 – 20	1,548	4.6	638	10.7
	> 20	1,113	5.5	674	9.2
Number of cattle purchased from test positive dairy herds in previous year-quarter	0	39,798	2.5	10,957	11.6
	1 - 10	823	8.0	811	9.4
	11 - 20	119	14.3	150	8.0
	> 20	72	18.0	147	6.8
Total number of source herds in previous year-quarter	0	29,579	2.4	7,976	11.3
	1	7,313	2.8	2,380	11.9
	> 1	3,920	4.8	1,709	10.9

(Continues on the next page)

(Table 2 continued)

Variables	Levels	Previously test negative herds N	Previously test negative herds % test negative to positive	Previously test positive herds N	Previously test positive herds % test positive to negative
Total number of test positive source dairy herds in previous year-quarter	0	39,798	2.5	10,957	11.6
	1	898	8.6	888	9.7
	> 1	116	16.4	220	5.5
Number of close-contact neighbors in C2a[1] or C3a[2] in previous year-quarter	0	22,509	1.8	6,096	11.2
	1 or more	683	3.8	630	5.9
Total number of close-contact neighbors in previous year-quarter	0	19,064	1.8	5,002	11.4
	1 or more	4,128	3.8	1,724	8.4
Breed	Large breed	40,358	2.8	13,357	11.3
	Jersey	6,161	1.6	743	19.5
Organic	No	43,091	2.6	12,337	12.2
	Yes	3,434	3.5	1,766	8.5
Season	Jan. to Mar.	11,730	2.0	3,940	13.8
	Apr. to Jun.	11,550	1.7	3,583	10.6
	Jul. to Sept.	10,092	2.7	2,813	10.2
	Oct. to Dec.	13,153	4.0	3,767	11.8
Region	N. Jutland (N)	3,611	1.9	873	15.8
	N. Jutland (S)	3,985	5.4	2,591	9.3
	N.W. Jutland	7,937	2.6	1,647	15.1
	East Jutland	7,585	1.3	4,533	9.7
	West Jutland	8,486	2.6	2,854	10.2
	South Jutland	7,888	4.2	1,011	17.0
	The Islands	7,033	1.3	594	20.7

[1] C2a = *Salmonella* category in the national surveillance program based on high antibodies

[2] C3a = *Salmonella* category in the national surveillance program based on laboratory confirmed salmonellosis due to *S*. Dublin
confirmed by positive bacteriological culture of *Salmonella* Dublin

Results

In the full data set of *Salmonella* test results a change from test negative to positive occurred in 2.6% of the available quarterly observations (n=46,525) from test negative dairy herds. A change from test positive to negative occurred in 11.7% of quarterly observations (n=14,103) available from test positive dairy herds.

After the data set was reduced to including the relevant period with data for all important variables, the dataset for test negative to positive changes had 21,007 observations and changes occurred in 2% of these observations. The dataset for test positive to negative changes had 6,168 observations, with changes occurring in 10% of these observations. Descriptive statistics including proportion of the two types of changes in herd status for each level of the categorical variables are given in Table 2.

Model 1: Change from test negative to positive indicating introduction of infection

The parameter estimates and significance levels in the model for becoming test positive after being test negative, which is indicative of new infection, are given in Table 3. The higher the number of C2a- or C3a-neighbor herds was in the local area (2 km radius around the herd), the higher ($P = 0.006$) was the risk of changing from test negative to positive. The risk of changing was also higher ($P < 0.0001$) if a herd had purchased animals from test positive herds in the previous YQ than if it had only purchased animals from test negative herds or not purchased animals at all. Because of high co-linearity between number of purchased cattle and number of source herds it was not possible to include both variables in the model simultaneously. When the number of test positive source herds was tested in this model instead of number of purchased cattle from test positive herds, this risk factor was also significant with increasing number of test positive source herds leading to higher odds of changing from *Salmonella* test negative to positive (results not shown).

Model 2: Change from test positive to negative indicating recovery from infection

Parameter estimates and significance levels of risk factors from the final model for changing from test positive to negative (indicative of recovery) are shown in Table 4. Odds ratios with 95% confidence limits for each main effect and examples from the interaction terms are shown in Table 5. The odds of changing from test positive to negative were influenced by the local cattle herd density (measured as number of neighbor herd manure disposal areas that included the herd at risk). Herds that did not have such close-contact neighbors in the previous YQ had higher odds of becoming test negative than herds with one or more close-contact neighbors. This variable was highly dependent on the size of the neighbor herds, because large herds would require larger areas for manure disposal than small herds. One other factor that described local cattle herd density was present in the final model, i.e. in the interaction between herd size and number of C2a- or C3a-neighbors in a 2 km radius. The calculation of the latter explanatory variable did not depend on the size of the neighboring herds. For medium and large herds, having 2 or more C2a- or C3a-herds in a 2 km radius lead to significantly smaller odds of becoming test negatve in the current YQ after having been test positive in the previous YQ. For small herds there was no effect of having such neighbors.

Table 3 Risk factors associated with a change in classification from test negative to positive (indicative of new infection) in the Danish surveillance program for *Salmonella* in 2003

Variables	Levels	β	S.E.	OR	95% CI for OR	*P*[a]
Intercept		-5.29	0.23			
Region	North Jutland (S)	1.60	0.24	5.0	3.1 – 8.0	< 0.0001
	North West Jutland	0.73	0.25	2.1	1.3 – 3.4	
	South Jutland	1.07	0.23	2.9	1.9 – 4.6	
	North Jutland (N)	0.46	0.32	1.6	0.9 – 2.9	
	West Jutland	0.75	0.24	2.1	1.3 – 3.4	
	The Islands	-0.16	0.29	0.9	0.5 – 1.5	
	Eastern Jutland	0	-	1.0	-	
Number of neighbors	> 2	0.55	0.15	1.7	1.3 – 2.3	0.006
classified as C2a[1] or C3a[2] in	2	0.26	0.17	1.3	0.9 – 1.8	
a 2 km radius in previous	1	0.27	0.12	1.3	1.0 – 1.7	
year-quarter	0	0	-	1.0	-	
Number of cattle	> 20	1.99	0.49	7.4	2.8 –19.3	< 0.0001
purchased from test	11 - 20	2.40	0.40	11.0	5.0 – 24.0	
positive herds in the	1 - 10	1.33	0.23	3.8	2.4 – 5.9	
previous year-quarter	0	0	-	1.0	-	
Herd size (per 10 animal increase)		0.024	0.005	1.024	1.02 –1.03	< 0.0001

[a] p-value estimated by the score statistics for Type 3 contrasts in the GEE analysis

[1] C2a = *Salmonella* category in the national surveillance program based on high antibodies

[2] C3a = *Salmonella* category in the national surveillance program based on laboratory confirmed salmonellosis due to *S*. Dublin

Dairy herds that did not have organic production systems had significantly higher odds of recovering to test negative compared with organic herds. Breed seemed to have an effect in large herds. For small herds there was no difference between the odds of changing to test negative, but for large herd (e.g. 300 cattle) the odds of changing to test negative were smaller for large breeds than for Jersey.

Table 4 Final model parameter estimates, standard errors (S.E.) and p-values for risk factors associated with a change from test positive to test negative (indicative of recovery) in the Danish surveillance program for *Salmonella* in dairy herds in 2003

Variables	Levels	β	S.E.	P[a]
Intercept		-2.49	0.39	
Region	North Jutland (S)	-0.17	0.21	0.003
	North West Jutland	0.21	0.21	
	South Jutland	-0.31	0.20	
	North Jutland (N)	0.18	0.23	
	West Jutland	-0.16	0.20	
	The Islands	0.40	0.25	
	Eastern Jutland	0	0	
Close-contact neighbors in the previous year-quarter	Yes	0.31	0.11	0.006
	No	0	0	
Breed	Large breed	0.39	0.32	0.2
	Jersey	0	0	
Organic	No	0.54	0.17	0.0003
	Yes	0	0	
Number of neighbors classified as C2a[1] or C3a[2] in a 2 km radius in previous year-quarter	> 2	0.14	0.26	0.4
	2	-0.12	0.33	
	1	-0.33	0.23	
	0	0	0	
Herd size (per 10 heads)		0.014	0.014	0.2
Herd size * breed	Large breed	-0.004	0.001	0.02
	Jersey	0	0	
Herd size * Number of neighbors classified as C2a[1] or C3a[2] in a 2 km radius in previous year-quarter	> 2 herds	-0.003	0.001	0.03
	2 herds	-0.001	0.002	
	1 herds	0.001	0.001	
	0 herds	0	0	

[a] P-value estimated by the score statistics for Type 3 contrasts in the GEE analysis

[1] C2a = *Salmonella* category in the national surveillance program based on high antibodies

[2] C3a = *Salmonella* category in the national surveillance program based on laboratory confirmed salmonellosis due to *S*. Dublin

Table 5 Odds ratios (OR) and 95% confidence intervals of the OR for risk factors for a change from test positive to negative (indicative of recovery) in the Danish surveillance program for *Salmonella* in dairy herds in 2003.

Variables	Levels		OR	95% CI of OR
Region	North Jutland (S)		0.9	0.6 – 1.3
	North West Jutland		1.2	0.8 – 1.9
	South Jutland		0.7	0.5 – 1.1
	North Jutland (N)		1.2	0.8 – 1.9
	West Jutland		0.9	0.6 – 1.3
	The Islands		1.5	0.9 – 2.4
	Eastern Jutland		1.0	-
Close-contact neighbors in the previous year-quarter	Yes		1.4	1.1 – 1.7
	No		1	-
Organic	No		1.7	1.2 – 2.4
	Yes		1	-
Herd size and Breed	Herd size	Breed		
	100	Large	1.0	(0.6 – 1.6)
		Jersey	1.0	-
	200	Large	0.7	(0.5 – 1.0)
		Jersey	1.0	-
	300	Large	0.5	(0.3 – 0.8)
		Jersey	1.0	-
Herd size and Number of neighbors classified as C2a[1] or C3a[2] in a 2 km radius in previous year-quarter	Herd	Neigbors		
	100	> 2 herds	0.9	(0.6 – 1.2)
		2 herds	0.8	(0.6 – 1.2)
		1 herds	0.8	(0.6 – 1.1)
		0 herds	1.0	-
	200	> 2 herds	0.6	(0.5 – 0.9)
		2 herds	0.7	(0.6 – 1.0)
		1 herds	1.0	(0.8 – 1.2)
		0 herds	1.0	-
	300	> 2 herds	0.5	(0.3 – 0.8)
		2 herds	0.7	(0.5 – 1.0)
		1 herds	1.1	(0.8 – 1.5)
		0 herds	1.0	-

[1] C2a = *Salmonella* category in the national surveillance program based on high antibodies

[2] C3a = *Salmonella* category in the national surveillance program based on laboratory confirmed salmonellosis due to *S*. Dublin.

Discussion

Herd classification and variables

S. Dublin was the most commonly isolated serotype in Danish cattle and it is known to usually persist longer in the herds than other serotypes (Boqvist and Vagsholm, 2005). Whereas it is likely that *S.* Dublin accounted for most herd infections in the present study period, the interpretation of the results extend to other cross-reacting *S. enterica* subsp. *enterica* serotypes that spread in similar ways to *S.* Dublin. The models for new infection and recovery of *Salmonella* infection in Danish cattle were based on herd test status determined by repeated bulk tank milk measurements. Thus, the outcome of interest was measured indirectly. There are no perfect diagnostic methods available to measure whether a herd is truly infected with *Salmonella* (Veling et al., 2002). With the method used in our study, the herd positive predictive value in the program was not perfect (estimated to 80%) and this may have had an impact on the results (Warnick et al., 2006). Whereas Veling et al. (2002) found the sensitivity of BTM ELISA to be small (54%) when basing herd classification on one single BTM sample, combining the results from four repeated samples as is done in the Danish *Salmonella* surveillance program seemed to improve the sensitivity (95%) (Warnick et al., 2006). Although detailed information on herds used for evaluation of the BTM ELISA (Nielsen and Ersbøll, 2005) raises confidence that risk factors identified in the study presented here are applicable to changes in herd infection status, potential effects of misclassification should be considered. For instance, a herd may become test positive after a rise in antibodies in the BTM when purchasing animals with antibodies from another herd without necessarily having introduced the infection into the herd. This would be more likely to occur, if the source herd was test positive. If this phenomenon was common in a large number of herds, the effect of purchase on becoming infected could have been overestimated in our analysis. Other studies, however, support the finding that the risk of becoming infected increases with purchase from other herds, and biologically it makes sense that this risk is mainly increased if the source herd is infected and thus test positive (Vaessen et al., 1998; van Schaik et al., 2002). Based on clinical experience with the BTM ELISA test as well as bacteriological culture results and other observations of test program herds, we believe the analysis identified factors likely to be associated with changes in *Salmonella* infection status of the herd and not just with changes in herd test results. Consistency with published results from other field studies strengthens this conclusion for the effect of purchasing cattle from infected herds and a number of other risk factors identified.

One possible improvement of the models could be to include infection history from YQ earlier than the most recent YQ. If a test negative herd had been infected with *Salmonella* (or had been test positive) within the last couple of years it was probably more likely to change to test positive again after re-infection from persistently infected animals or surviving bacteria in the environment (Wray et al., 1989; House et al., 1993). This should preferably be assessed using a dataset containing data from a longer period than was available for this study. Another variable that could not be included in this study was the concurrent infection with other diseases, such as metabolic diseases, liver fluke infestation or viral infections that may reduce the resistance to *Salmonella* in the herds (Aitken et al., 1981; Vaessen et al., 1998).

Model results

The number of presumably infected (C2a or C3a) neighbor herds was a significant risk factor common for both models. The higher this number, the more likely change from test negative to positive was to occur and the less likely test positive herds were to become test negative between two YQ. The results are supported by another study (Wedderkopp et al., 2001). In that study it was found that the risk for a dairy herd to change infection status was associated with the BTM status of the nearest neighbors and the prevalence of seropositive herds in the geographic area.
Herd size was also included in both models. The bigger the herd the more likely it was to change from test negative to positive (Model 1). Changing from test positive to negative, the effect of herd size was less clear. Large herds consisting of large breed or having C2a or C3a neighbors in a 2 km radius were less likely to recover, whereas the breed and neighbor factors were not found important for small herds. Herd size is often found a risk factor for infectious diseases (Vaessen et al., 1998; Warnick et al., 2001). Herd size, however, may be an indirect measure of management. In this study, we did not have access to data to investigate possible underlying reasons for the effect of herd size. In two studies in which there were more elaborate details of management available for the model, no association between *Salmonella* fecal shedding and herd size was detected (Fossler et al., 2005a; Fossler et al., 2005b).

The number of purchased cattle from test positive herds was associated with becoming infected. Other studies have found purchase of live animals a significant risk factor for introduction of *Salmonella*-infection to the herd (Wray et al., 1990; Wray et al., 1991; Vaessen et al., 1998; van Schaik et al., 2002). In the surveillance program, purchase of cattle from C2a-dairy herds lead to automatic classification in C2b for 3 mo from the day of purchase. This was a restriction implemented to attempt to minimize transmission between herds. C2b was an indicator that the herd had been involved in high risk activity (contact to assumed infected herds) and other herds were less likely to purchase cattle from the herds in this category. The fact that purchase from test positive herds was found a significant risk factor for changing to test positive supports this control strategy. Whether the length of the automatic classification period based on trade could be improved was not investigated further in this study.

Organic production was only associated with persistence of test positive results, not with becoming test positive. This may be because of regulations about management procedures in organic herds that allow for easy spread of infection from dams or calving environment to newborn calves and calving cows such as the practice of leaving the calf with the dam up to 3-4 d after birth, less aggressive antibiotic usage, no preventive treatments allowed and possible differences in feeding strategies (Fossler et al., 2005a; Fossler et al., 2005b).

In the final model there was no association between calf mortality in the previous YQ and a change in test results. Data quality for the variable “calf mortality” was, however, not ideal. The variable was constructed as the number of dead calves out of all calves born. The calf mortality percentage varied dramatically because of the small number of calves born per YQ in some herds. It is likely that increased calf mortality more often would follow rather than precede the change of *Salmonella* status. For future studies, it should be investigated how to construct a reliable calf mortality or calf morbidity variable and to consider how to include it in a model as it is likely to be associated with many outbreaks and re-infections with *S.* Dublin and other types of *Salmonella* in dairy herds and it may be useful as an early indicator of new or re-infection with *Salmonella*.

Conclusions

The results have implications for controlling *Salmonella* infection in cattle herds. Herd owners should be aware of the infection risk when purchasing of new livestock from an infected herd and the risk of having infected neighbors. High external bio-security is necessary in such herds. There is a need to inform organic farmers, herds in high cattle density areas and herds with test positive neighbors how to control and eradicate *Salmonella*. High internal *and* external bio-security is required, not just control of within-herd transmission that tends to be the main focus in infected herds. The results provided support for trade restrictions upon purchase of cattle from C2a herds in the surveillance program, as there was in fact a high risk of infection associated with this behavior.

The association between calf mortality (or calf morbidity) and *Salmonella* infection should be investigated using higher quality data than was available in this project. This would help determine if calf mortality and/or calf morbidity is useful as an early warning of new infection in herds that have not yet had an increase in BTM ELISA.

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PAPER VII

Survival analysis of factors affecting incidence risk of *Salmonella* Dublin in Danish dairy herds during a 7-year surveillance period

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Survival analysis of factors affecting incidence risk of *Salmonella* Dublin in Danish dairy herds during a 7-year surveillance period

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Abstract

A national surveillance programme for *Salmonella* Dublin, based on regular bulk-tank milk antibody screening and movements of cattle, was initiated in Denmark in 2002. From 2002 to end of 2009 the prevalence of test-positive dairy herds was reduced from 26% to 10%. However, new infections and spread of *S.* Dublin between herds continued to occur.

The objective of this study was to investigate factors affecting incidence risk of *S*. Dublin infection in Danish dairy herds between 2003 and 2009. Herds were considered at risk when they had been test-negative for at least four consecutive year-quarters (YQs), either at the start of the study period or after recovery from infection. Survival analysis was performed on a dataset including 6,931 dairy herds with 118,969 YQs at risk, in which 1,523 failures (new infection events) occurred. Predictors obtained from register data were tested in a multivariable, proportional hazard model allowing for recurrence within herds.

During October to December the hazard of failures was higher (hazard ratio HR=3.4, P=0.0005) than the rest of the year. Accounting for the delay in bulk-tank milk antibody responses to *S*. Dublin infection, this indicates that introduction of bacteria was most frequent between July and October. Purchase from test-positive cattle herds within the previous 6 months was associated with higher hazard of failures (HR=2.5, P<0.0001) compared to no purchase and purchase from test-negative herds. Increasing local prevalence, herd size and bulk-tank milk somatic cell counts were also associated with increasing hazard of failures. The effect of prior infection was time-dependent; the hazard of failures was reduced following a logarithmic decline with increasing time at risk. The hazard was markedly higher in herds with prior infections the first year after becoming at risk again, and then approached the hazard in herds without known prior infections 2-3 years after becoming test-negative. This showed that herds with prior infections need to maintain a high level of biosecurity for at least 3 years after becoming test-negative for *S*. Dublin to prevent recurrence. Furthermore, general recommendations for dairy herds wishing to protect their herds against *S*. Dublin include avoidance of purchase from and contact to test-positive herds. Large herds, herds with test-positive neighbours and herds with high somatic cell counts need to obtain and maintain a high level of biosecurity.

Introduction

Salmonella enterica subsp. *enterica* Dublin (*S*. Dublin) has been a cause of concern in the Danish cattle industry for more than a decade due to a relatively high prevalence at approximately 26% seropositive dairy herds in 2003 (Anonymous, 2004). Infected herds often experience economic losses and welfare consequences such as increased mortality, morbidity, abortions and reduced milk yield (Richardson and Watson, 1971; Visser et al., 1997; Nielsen et al., 2010, 2012c). Furthermore, rare but serious human infections occur and are mainly attributed to consumption of beef (Helms et al., 2003).

Therefore, a national surveillance programme for *S*. Dublin was initiated in the Danish cattle population in 2002. Dairy herds are classified into infection categories based on screening of bulk-tank milk antibodies four times per year and movements of cattle between herds with different test statuses (Warnick et al., 2006). The data collected through the surveillance programme from 2002 and onwards have been stored in the Danish Cattle Database. These data provide a unique source of information for investigation of factors affecting the time to infection with *S*. Dublin in Danish dairy herds. Furthermore, the extended period of time from which data are available allows for analysis of changes in the effects of relevant risk factors over time during the surveillance period. In 2007, a control campaign mainly consisting of regionally organised encouragement of farmers in test positive herds, information material and projects to demonstrate the effect of control strategies were initiated by the Danish Cattle Federation in September 2007 (Nielsen et al., 2006). The prevalence of test-positive dairy herds was reduced to 10% by end of 2009. However, new infections still occurred and continued to lead to spread of *S.* Dublin between cattle herds leading to potential failure to reach the aim of the programme: to eradicate the infection from the cattle population by end of 2014.

One previous study investigated risk factors associated with switching from test-negative to test-positive between consecutive quarters within one year (indicative of a new infection) (Nielsen et al., 2007). It was found that season, increasing herd size, increasing number of purchased animals from test-positive herds and increasing number of test-positive neighbouring farms in the previous three months all significantly increased the risk of becoming test-positive (Nielsen et al., 2007). However, that study was based on data from one year, 2003, just after the surveillance programme was initiated. It was therefore impossible to study the effect of time and changes in the surveillance programme on the risk of becoming test-positive and the effect of the risk factors identified in the study. Such information would be useful for the cattle industry and veterinary authorities to support future decisions on adjustments to the surveillance and control programmes.

The objectives of the present study were: 1) to investigate the incidence risk of becoming test-positive for *S*. Dublin (indicative of new infection or recurrence events) in Danish dairy herds over time from beginning of 2003 to end of 2009, 2) to investigate predictors and time-dependency of predictors for time to infection, and 3) to investigate if and when herds that became test-negative after a test-positive period reached the same or lower hazard of becoming test-positive as herds that had been test-negative from the beginning of the surveillance programme. The third objective would provide new insights into the risk of becoming test-positive in the surveillance programme that was attributable to re-infection of livestock from the immediate environment or latent carriers in the herd rather than introduction of new infection from outside of the herd. Such insight is requested by the Danish veterinary authorities to guide future changes to legislation concerning management practices aimed at *S*. Dublin eradication.

Materials and methods

Sampling and categorisation of herds

All active Danish dairy herds were sampled every three months in the surveillance programme for *S*. Dublin from January 2002 and onwards (Anonymous, 2004). The bulk-tank milk samples were analysed for antibodies directed against *S*. Dublin lipopolysaccharide O-antigens using an in-house ELISA at Steins Laboratory in Ladelund from January 2002 to March 2005, the national reference

laboratory at the Veterinary Institute, Technical University of Denmark, Copenhagen from April 2005 to March 2008 and Eurofins Steins Laboratory in Holstebro from April 2008 until the data for analysis were extracted in April 2010. The dataset contained a variable indicating which laboratory analysed the samples. Preliminary analyses did not suggest differences in ELISA results between the laboratories of relevance for this study. Furthermore, this surveillance system was reasonably stable against test day and batch variations, and laboratory changes because the status of the herd was calculated based on four bulk-tank milk samples over a 1-year period. Furthermore, the laboratory running the samples were continuously monitored by the National Veterinary Institute (the national reference lab) for eventual diversions from the quality assurance criteria. Therefore, test day, batch and other test or laboratory specific information was not included in the analyses.

In the surveillance programme, herds have their classification re-evaluated every time new bulk-tank milk ELISA results become available. For this study the available data were compiled into year-quarterly (YQ) herd test statuses. The bulk-tank milk ELISA results were reported as background corrected optical density values (ODC%) indicating the level of antibodies in the assay (Nielsen and Ersbøll, 2005). For each YQ, herds were categorised as test-negative if the average of the latest four measurements was <25 ODC%, and the difference between the current measurement and the average of the previous three was <20 ODC%. Otherwise, the herd was categorised as test-positive. This categorisation method was evaluated in a previous study, which found that the herd sensitivity was 0.95 independent of prevalence, and the herd specificity varied from 0.83 at an underlying prevalence of 50% to 0.98 at 2% infected herds (Warnick et al., 2006).

In the preparation of a dataset for survival analysis, herds represented with *S*. Dublin classification results from less than four YQs were excluded. This meant that 1,910 YQs were removed from the dataset, leaving 191,998 YQs from 7,732 dairy herds with test statuses available for further data management. The mean, median and maximum numbers of YQs per herd were 25, 33 and 33, respectively. Based on the combination of the test status of the current and the previous four YQs, the failure event, “new infection”, was created. This variable indicated whether a herd shifted test status from negative to positive between two YQs. However, the herd had to be test-negative for four consecutive YQs prior to the shift to test-positive in order to be included as having a failure in the given YQ. This was done to increase the probability that the failure truly represented a new introduction of *S*. Dublin infection to the herd rather than representing re-infection of live-stock on the farm from an environmental, or low prevalent persistent infection leading to new spread of infection followed by increased antibody levels in the bulk-tank milk. This criterion meant that the first four YQs for each herd were only used to determine the initial status of the herd. A count distribution of all 32 possible combinations of test-statuses in five consecutive YQs showed that 724 observations (0.5%) included one, two or three consecutive test-negative YQs followed by a test-positive YQ in the current YQ, but these were not considered new infections due to the four consecutive negative YQ criterion mentioned above (e.g. neither of the patterns 10001 and 10101 - with 0 being test-negative and 1 being test-positive and the most recent YQs test result to the right - were indicative of a new infection). The combination which was considered indicative of new infection consisted of four consecutive test-negative YQs followed by a test-positive YQ (i.e. 00001). This occurred in 1,553 (1%) of the YQs. The rest of the combinations included 121,924 (75.7%) YQs with five consecutive test-negative YQs in which the herds were at risk of failures, 21,490 (13.3%) with five consecutive test-positive YQs and 15,379 (9.5%) with other mixed patterns where herds could not be considered at risk due to the described criteria.

The study period was further restricted to 1st of January 2003 to 31st of December 2009 for statistical analysis, because in these seven years all YQs were represented and the herds could be at risk of new infections. This reduced the total number of observations in Dataset A to 157,181 YQs from 6,931 herds including 118,425 YQs at risk, 1530 YQs with new infection events and 37,226 YQs that were part of gaps, e.g. YQs in which herds were test-positive (without it being a failure) but not yet at risk.

To be able to explore the effect of the criteria used to determine when a new infection occurred, two other datasets were constructed in which four consecutive test-negative YQs were still required before a failure could occur, but where individual test-positive YQs were not considered truly positive unless they were part of a string of two or more test-positive YQs, only interrupted by test-negative periods of less than four YQs. This combination of criteria was used to decrease the number of false positive herd classifications. There were 941 YQs with new infection events combined with all relevant predictors. In the first alternative dataset (Dataset B), single test-positive YQs were set to missing and all other variables were kept the same as in Dataset A. In the second alternative dataset (Dataset C), single test-positive YQs were set to negative assuming these were due to false positive bulk-tank milk antibody measurements. The time at risk and number of prior infections was recalculated accordingly meaning that the number of prior infections became lower and the time at risk was longer in herds that had single test-positive YQs. Examples of the coding of YQ test results, time at risk, failure events, gaps and number of prior infections in all three datasets are illustrated in Table 1.

Table 1 Examples of coding of the status for each year-quarters (YQ) regarding time at risk, with failures and gaps for one dairy herd in three difference datasets (A, B and C) used for survival analysis of *S*. Dublin introduction to dairy herds based on original Danish surveillance programme test status data

	YQ[a] number	20021	20022	20023	20024	20031	20032	20033	20034	20041	20042	20043	20044	20051	20052	20053	20054	20061	20062
Datasets (Failure criteria)	Test status in YQ	-	-	-	-	-	-	+	-	-	-	-	-	+	+	+	+	+	-
A (4 neg YQ+1	Coding in datasets[c]	G	G	G	G	0	0	1	G	G	G	G	0	1	G	G	G	G	G
Time at risk in YQs		.	.	.	.	1	2	3	.	.	.	.	1	2	.	.	.	.	.
# of prior		.	.	.	.	0	0	0	1	1	1	1	1	1	2	2	2	2	2
B (4 neg YQ+2		G	G	G	G	0	0	G	G	G	G	G	0	1	G	G	G	G	G
Time at risk in YQs		.	.	.	.	1	2	.	.	.	.	.	1	2	.	.	.	.	.
# of prior		.	.	.	.	0	0	0	0	0	0	0	0	0	1	1	1	1	1
C (4 neg YQ+2		G	G	G	G	0	0	0	0	0	0	0	0	1	G	G	G	G	G
Time at risk in YQs		.	.	.	.	1	2	3	4	5	6	7	8	9	.	.	.	.	.
# of prior		.	.	.	.	0	0	0	0	0	0	0	0	0	1	1	1	1	1

After reduction of the datasets for analysis, there were 6,944 herds with 1,523 failures in 119,955 YQs at risk in Dataset A. In Datasets B and C there were 6,927 herds that contributed to the analysis with time at risk. There were 941 failures in 118,387 YQs at risk in Dataset B and 941 failures in 121,345 YQs at risk in Dataset C.

Construction of risk factors from register data

Routinely collected data on geographical location of herds, movement of animals, production practices and milk quality control data were extracted from the Danish Cattle Database from January 1st, 2002 to April 1st, 2010. The following variables were constructed for each YQ for further analysis:

Year quarter (YQ): YQ were based on the months January to March, April to June, July to September and October to December. The distributions of number of dairy herds at risk of new infection and incidence risk for each YQ in the study period are shown in Figure 1.

Season: Due to the year-quarterly sampling scheme season was set to follow the sampling periods equal to the months used to define the YQs.

Farming type: Information about registered organic farming was obtained from the Danish Plant Directorate and merged with data from the Danish Cattle Database. Because it was not accurately recorded when herds switched to organic farming practices and back from organic to conventional farming practices, this variable indicated whether or not a herd was recorded as having used organic farming practices during the study period.

ParaTB: A voluntary control programme for paratuberculosis was initiated in Denmark in March 2006. Herds participating in this programme were encouraged to optimise biosecurity procedures to prevent introduction and spread of infection. This might also affect the risk of introduction of *Salmonella*. Therefore, this variable indicating whether herds at risk of new infection were recorded as part of the paratuberculosis control programme was included in the analysis.

Purchase: Based on movement data this variable indicates whether herds during a 6-month period prior to the current YQ had purchased no animals at all, only animals from test-negative herds or if the purchases had included purchase of animals from test-positive herds.

Herd size: The herd size was recorded as the total number of animals averaged across monthly counts within each YQ. This meant that the variable was continuous rather than discrete.

Prior infection: Indication of whether the herd had been test-positive earlier on in the surveillance programme counting from January 2002. Note the difference in counts of prior infections between the three datasets as illustrated in Table 1.

YQs at risk: The duration of time at risk was counted as the number of consecutive test-negative YQs prior to the current YQ minus the four test-negative YQs required to ensure that the herd was truly at risk of infection. Note the difference in counts of YQs at risk between the three datasets as illustrated in Table 1.

Local herd prevalence: This variable specified the number of test-positive cattle herds (both non-dairy and dairy herds) in a 5 km radius around each study herd divided by the total number of herds for each YQ.

Local herd prevalence: This variable specified the number of cattle present in dairy and beef herds in a 5 km radius around the study herd for each YQ.

LogSCC: The logarithm of the average bulk-tank milk somatic cell count divided by 1,000 measured during the YQ.

Some of these variables are to some degree proxies for internal biosecurity (e.g. *Farming type*, *ParaTB*, *LogSCC*). Others are related to external biosecurity (e.g. *Local herd prevalence, Local herd prevalence* and *Purchase).*

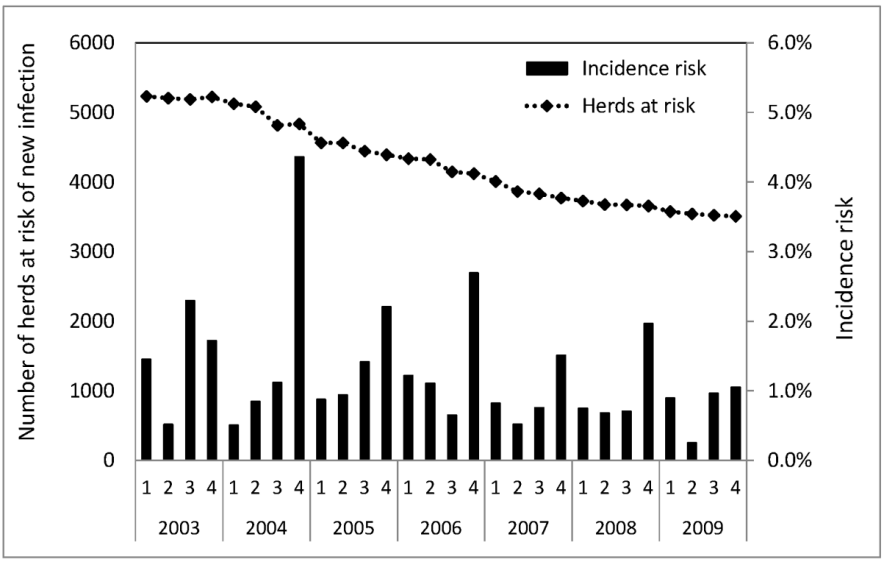

Figure 1 The stippled line relates to the left y-axis and shows the number of herds at risk of new *S*. Dublin infection (i.e. having at least four consecutive test-negative year-quarters prior to the current). The bars relate to the right y-axis and show the *S*. Dublin incidence risks in each year-quarter (YQ) according to bulk-tank milk surveillance data from all Danish dairy herds from 2003 through 2009.

Statistical analysis

All categorical variables were checked for distribution of observations (i.e. number of YQs and number of herds represented) within each level of the variable. A semiparametric, proportional hazard model allowing multiple failures to occur in the same herd was used for the time to event analysis. We used the Anderson-Gill model, and included a time-varying covariate for prior infections to relax the assumption that the hazard of recurrence is assumed to be independent of previous events (Dohoo et al., 2009). The time to event was counted from when the herd became at risk, i.e. starting after four consecutive test negative year-quarters either at the beginning of the sampling period or after becoming test negative after a test positive period. The analysis was performed in STATA® IC/11 (StataCorpLP, College Station, Texas, USA). YQs in which the herds could be considered at risk of having a failure (n= 119,955 in Dataset A) and those in which they actually had a failure (n=1,523) were included in the analysis. YQs not included in the analysis (n=37,226) were considered gaps either due to uncertain herd test status in that YQ according to the criteria for being at risk, or because the herd was test-positive and therefore not at risk of becoming infected. Efron's method was used to handle ties in the data (i.e. multiple new infection

events in the same YQ). Reasonable functional forms of continuous predictors were determined by evaluation of lowess smoothed graphs of Martingale residuals for the original variable, and if necessary appropriate transformations of the original variable were performed (Dohoo et al., 2009).

A stepwise forward selection procedure was used to test the main effects of the predictors including all possible two-way interactions. Evaluation of time-varying effects was performed at the end of the modelling procedure. Due to the size of the dataset, effects were included in the final model at a 1% significance level. The assumption of proportional hazards was evaluated graphically for the categorical variables and by graphical and statistical test evaluation of Schoenfeld residuals for continuous variables included in the final model. These procedures evaluated whether or not there was evidence that some hazard ratios were not constant (i.e. changed over time) (Dohoo et al., 2009). The assumption of independent censoring was evaluated by sensitivity analysis comparing scenarios with changed positive and negative correlations between censoring and new infection events. Outliers were checked for by plots of deviance residuals vs. time and influential points by plots of score residuals vs. time.

Results

There were 6,944 herds in Dataset A that contributed time at risk to the analysis. Out of these, 5,559 herds did not experience any failures between 2003 and 2009, 1,244 had one, 137 had two and 4 herds had three failure events (i.e. 1,530 failure events in total). However, only 1,523 of the failure events were used in the final model parameter estimation because of missing data for somatic cell counts for seven of the events. The overall incidence risk was 1.3%. The distributions of new infection events (i.e. the incidence risk) for each level of the categorical variables are shown in Table 2. Descriptive statistics of all continuous variables and the discrete variable “YQs at risk” are shown Table 3. The functional forms of the continuous predictors were evaluated to be reasonable as linear effects except for the variable number of “YQs at risk”, which was used to model the time-varying effect of prior infection. The best model fit (i.e. the model with the lowest log likelihood) was obtained using the logarithm of number of YQs at risk to model the time-varying effect of prior infection.

Results of the final proportional hazards survival model are provided in Table 4. Generally, herds with prior infection had higher hazard of failures. However, the effect interacted with the local prevalence, so that the higher the local prevalence the lower the effect of prior infection was. This relationship was complicated by the fact that there was a non-linear (logarithmic) time-varying effect of time at risk. Figure 2 provides plots of hazard for failures over time between beginning of 2003 and end of 2009 for different settings of the risk factors determined as statistically relevant in the final model. The hazard function plots show a pattern with increasing hazard over the first 2½ years (from YQ 1 to 10 representing year 2003 to mid 2005) and decreasing hazard for 2 years (from YQ 11 to 18 representing mid 2005 to mid 2007) followed by a stable period for 1½ years (mid 2007 through 2008) and an accelerated decrease in hazard during the last year (from YQ 24 to 28 representing 2009).

Table 2 Descriptive statistics of categorical risk factors and *S.* Dublin incidence risks for each level of the risk factors based on register data from all Danish dairy herds between 2003 and 2009 (Dataset A).

Risk factors		Number of herds represented in each risk factor level[a]	Number of YQs at risk	Incidence risk
Season				
	January-March	6,759	30,570	0.9%
	April-June	6,648	30,258	0.7%
	July-September	6,526	29,621	1.2%
	October-December	6,488	29,506	2.3%
Farming type				
	Organic	660	12,082	1.5%
	Conventional	6,284	107,873	1.3%
Period				
	Surveillance	6,644	75,600	1.5%
	Control	4,538	44,355	0.9%
ParaTB				
	Not in PTB-programme	6,851	106,251	1.3%
	Part of PTB-programme	1,336	13,704	0.9%
Purchase				
	From test-positive herds	1,284	3,478	4.2%
	From test-negative herds	4,701	38,190	1.3%
	No purchases	6,343	78,287	1.1%
Number of prior infections				
	3	4	40	N/A[b]
	2	137	1,738	2.2%
	1	1,244	25,161	2.1%
	0	5,559	93,016	1.1%

The time-dependent effect of prior infection over time at risk is illustrated in Figure 3. In this figure, prior infection, local prevalence and time at risk vary, while parameter estimates for the other categorical explanatory factors are set to those relevant when no animals were purchased during the previous 6 months and season was the high risk season, October to December. The continuous variables were set to mean values (i.e. *LogSCC*=5.37 and herd size=208) in Figure 3.

Table 3 Descriptive statistics of continuous variables tested in a survival analysis of predictors for *S.* Dublin introduction to Danish dairy herds between 2003 and 2009 (Dataset A)

Variables	Min	Mean	Median	Std	Max
YQs at risk	1	11.4	10	7.7	28
Herd size[a]	0.3	208	187	138	3,202
Local herd prevalence	0	0.097	0.075	0.084	0.5
Local cattle density	1	4,520	4,218	2,488	13,762
LogSCC[b]	3.2	5.4	5.5	0.3	7.3

[a] Average herd size based on monthly recordings of all cattle in the herd across each YQ

[b] The natural logarithm of the bulk-tank milk somatic cell count in thousands averaged over the YQ

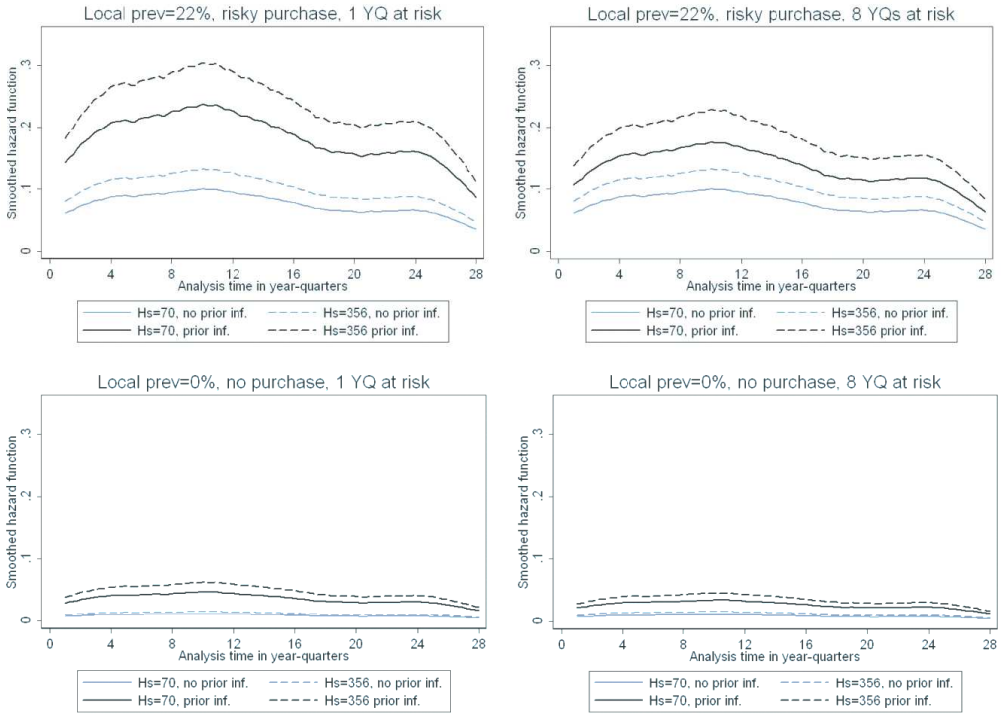

Figure 2 Smoothed Cox proportional hazard functions for new infection events in dairy herds in the October to December seasons between 2003 and 2009 in the Danish surveillance programme for *S.* Dublin for four different low and high risk scenarios (i.e. prior and no prior infection, herd size set to the 10th percentile (70 animals) and the 90th percentile (356 animals), no purchase and risky purchase, local prevalence set to the 10th percentile (0%), and the 90th percentile (22%), and 1 and 8 YQs at risk, respectively). LogSCC is set to 5.37 (median corresponding to 215,000 SCC). Analysis time on the x-axis goes from 1 (first year-quarter of 2003) to 28 (last year-quarter of 2009).

Being enrolled in the Danish voluntary paratuberculosis control programme was not significant at the 1% level, nor was the variable a confounder, so the predictor was removed from the final model even though the results could be interpreted as a tendency towards lower hazard of new *Salmonella* infection, if the herd was enrolled in the paratuberculosis programme (coefficient: -0.218, HR=0.8 (95%CI: 0.7-1), *P*=0.04).

The model fit as assessed by plots of Shoenfeld residuals for continuous variables did not raise concerns (data not shown). The assumption of independent censoring was evaluated to be reasonable by sensitivity analyses of correlations between censoring and new infection events, and we did not find influential outliers in the data.

The final models, based on Dataset B and Dataset C, were similar to the final model for Dataset A with regard to direction and significance of parameter estimates (data not shown). In general, the significant parameter estimates were 0-27% further away from zero in the final model based on Dataset B, and 3-37% further away from zero using Dataset C.

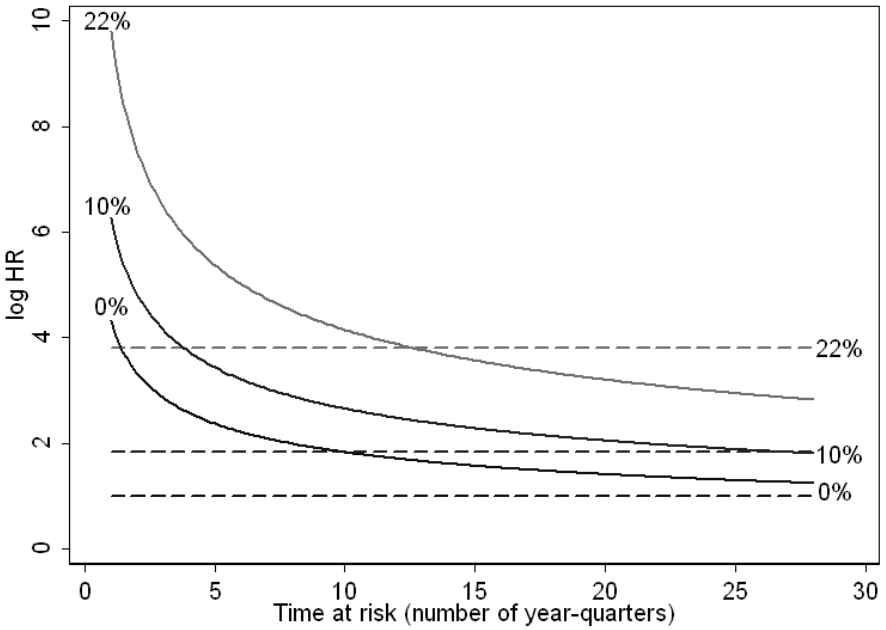

Figure 3 Model predicted log hazard ratio (log HR) for the effect of prior infection on the hazard of *S*. Dublin introduction to dairy herds as a function of time at risk (number of year-quarters at risk) at three different local prevalence levels (0%, 10% and 22%). The programme was initiated in 2002 and four test-negative YQs were required before herds were considered at risk, so in beginning of 2010 herds could have been at risk for up to 7 years (28 YQs). The horizontal dashed lines indicate the baseline hazard (no prior infection) for the given local prevalence group.

The model predictions based on these two models were very similar to those based on the final model using Dataset A, except that during the first year after becoming at risk the hazard was markedly higher than shown in Figure 3 indicating that when removing potentially false positive YQs from the analysis the effects of the predictors including prior infections became more pronounced.

Discussion

Model results

A proportional hazard survival model allowing for recurrence of failure events within herd was used to investigate the time to infection with *S*. Dublin in Danish dairy herds measured as changes from test-negative to test-positive between two year-quarters from 2003 through 2009 in the national surveillance programme for *S*. Dublin. Overall, new infection events occurred in 1.3% of the 119,955 YQs at risk. In spite of large seasonal differences (from 0.2% to more than 4% incidence risk per YQ), the hazard of new infection events reduced over time as illustrated in Figure 1.

The periodically varying hazards illustrated in Figure 2 were clearest and the hazards were highest for herds with high local prevalence, prior infection, short time at risk and large herd size (Figure 2). For herds located in an area with low local prevalence that did not purchase any cattle from other herds, the hazard of new infection events was low throughout the study period (<0.08). For herds with no prior infection in low prevalence areas the predicted hazard was close to zero. These results are supported by previous studies showing an increased risk of becoming infected with *S*. Dublin in cattle herds with increasing number or density of infected neighbour herds (Nielsen et al., 2007; Ersbøll and Nielsen, 2008, 2011). Overall cattle density was correlated with local prevalence and could therefore not be in the model together with local prevalence. It was a significant predictor if replaced into the model instead of local prevalence. Farming type was not associated with new infection events in this study suggesting that organic dairy herds had similar hazard of introduction of *Salmonella* into the herd as conventional herds.

Season was identified as a highly significant risk factor for new infection events with October to December being the YQ with highest hazards. Keeping in mind the delay in bulk-tank milk serological reactions and measurements this indicates that the YQ with the most herds becoming infected with *S*. Dublin (or other serotypes that can cross-react with the antigen used in the test) would be July to October. This pattern has been confirmed in two other studies from Sweden and United Kingdom with similar climate and cattle production systems (Carrique-Mas et al., 2010; Lewerin et al., 2011). However, the reasons for this clear temporal pattern remain speculative. One previous study of salmonellosis in cattle in Denmark suggested that high temperatures in June to August were associated with a higher number of *Salmonella* outbreaks in cattle herds than when average temperatures were lower in those months (Steffensen and Blom, 1999).

Purchase from test-positive cattle herds, increasing herd size and increasing somatic cell counts in bulk-tank milk were clear risk factors for new infection. None of these factors had significant time-varying effects when tested in the model, so it was concluded that the effects of these factors did not change over time in the programme. The first two factors have been found in other studies and are likely to be related to exposure from external sources of infection (van Schaik et al., 2002; Allerberger et al., 2003).

Table 4 Parameter estimates (β), standard error (S.E.), hazard ratios (HR), 95% confidence intervals for HRs and significance level (*P*) in the final proportional hazards survival model for *S*. Dublin introduction in Danish dairy herds 2003-2009.

Predictors	Estimate (β)	S.E.	HR	(95%CI of HR)	*P*
Season					0.0005
January-March	0.196	0.380	1.2	(0.6-2.7)	
April-June	0	-	1	-	
July-September	0.512	0.346	1.7	(0.8-3.3)	
October-December	1.223	0.350	3.4	(1.7-6.8)	
Purchase					< 0.0001
No purchases	0	-	1	-	
From test-negative herds	0.021	0.059	1.0	(0.9-1.1)	
From test-positive herds	0.919	0.096	2.5	(2.1-3.0)	
Prior infection					< 0.0001
No	0	-	1	-	
Yes	1.464	0.193	4.3	(3.4-5.5)	
Local herd prevalence[a]	0.608	0.034	1.8	(1.7-2.0)	< 0.0001
Prior infection * local herd prevalence[a]	-0.236	0.057	0.8	(0.7-0.9)	< 0.0001
Herd size (per 100 increase)	0.103	0.021	1.1	(1.1-1.2)	< 0.0001
LogSCC[c] (per log unit increase)	1.009	0.193	2.7	(1.9-4.0)	< 0.0001
Time varying effect					
Prior infection * log(YQs at risk	-0.373	0.048			< 0.0001

[a] Effects including local herd prevalence was estimated for 10% increases

Herd size is likely to be associated with herd susceptibility patterns that are favourable for *S*. Dublin spread, whereas somatic cell count is likely to be related to hygiene and general management practices in the herds that might influence the probability that transmission of infection will occur upon exposure (Nielsen et al., 2012a). We found a borderline significant tendency towards a positive effect of participating in a paratuberculosis control programme. The programme was initiated in 2006, so the effect of this programme might not have had its full effect yet in the study period of the present study. Hence, further investigations into the potential benefits of one disease control programme on other disease control programmes are encouraged.

Effect of prior infection

Not surprisingly, prior infection was a clear predictor for new infection events. *S*. Dublin survives well in the environment, e.g. in dried faecal matter, stored manure or slurry, and may lead to re-infection of the livestock (Findlay, 1972; Forshell and Ekesbo, 1993). Furthermore, *S*. Dublin is known to be able to produce persistently infected carrier animals that may shed bacteria intermittently, or they may be latently infected and become reactivated and lead to renewed spread of infection among susceptible cattle (Wray and Snoyenbos, 1985; Wray et al., 1989; House et al., 1993). According to Jordan et al. (2008), the time from recovery of a herd from *S*. Dublin infection to when the bulk-tank milk antibody levels decreased to below the cut-offs to become

test negative in the surveillance programme was 0 to 810 days with the most likely estimated being 180 days. We required 365 days (four YQs) for a herd to be considered at risk of new infection, meaning that we may have misclassified an unknown proportion of re-infections as new infection events. We found that the effect of prior infection reduced with time at risk. The function describing the decline in the effect of prior infection best included the logarithm of time at risk, which lead to the pattern illustrated in Figure 3. Initially after becoming at risk, the hazard was high but it rapidly declined over the first 2-3 years at risk and then levelled out to a slow decline from the third year at risk. This is likely due to a reduction in the risk of re-infections or renewed increases in bulk-tank milk antibodies in herds with persistent, low prevalent infections.

These results suggest that the surveillance had an effect, not only on the prevalence but also on the incidence risk, in particular from 2005 and onwards. The control programme that was initiated in October 2007 probably had the highest effect after 2008. This may be related to the fact that herds actively controlling ongoing infection within the herd were infectious to other herds for a shorter time span leading to fewer exposures to other herds, but it may also be related to the generally decreasing prevalence leading to fewer exposures of herds at risk (Jordan et al., 2008). It might also be explained by a higher degree of cautious purchase behaviours in test-negative herds. Such change in farmer behaviour might be a consequence of a centrally coordinated information campaign increasing awareness of the risk of purchasing *S*. Dublin infected cattle from test-positive herds (Nielsen et al., 2007; Bergevoet et al., 2009). Finally, the effect may be due to lower risk of recurrence of *S*. Dublin in herds with prior infections due to more optimal control procedures focusing on management changes to improve barriers against transmission of the bacteria (Hardman et al., 1991), and test-and-cull procedures to clear the herd of potential carrier animals (Bergevoet et al., 2009; Nielsen and Nielsen, 2011; Nielsen and Dohoo, 2011). This explanation is likely, because in 2007 and 2008 the centrally coordinated control campaign included financially supported farmer experience groups assisted by cattle consultants and veterinarian facilitators in all high prevalence regions of Denmark. Approximately 50% of the owners of test-positive dairy herds participated in an experience group for a period of at least 3 months. This is likely to have improved the effect of their control efforts and reduced the risk of recurrence in their herds.

Effect of herd classification criteria

The definition of new infection events (failures) in the dataset was based on interpretation of repeated *Salmonella* serogroup D specific antibody measurements in bulk-tank milk measured on a 3-monthly basis. This is an indirect measure of *S.* Dublin infection in the cattle population of the herds, and we cannot be certain that the changes from test-negative to test-positive in the surveillance programme classifications were actually due to introduction of *S*. Dublin to the herd. First of all, false positive reactions do occur. Warnick et al. (2006) estimated that approximately 20% of test-positive dairy herds had a prevalence of less than 5% seropositive cattle in the herd indicating that they were most likely not infected with *S*. Dublin or other serotypes of *Salmonella* that might cross-react with the antigen used in the test (Konrad et al., 1994). That study, however, included both newly infected herds, persistently infected and recovering dairy herds at an underlying true prevalence of 15% infected dairy herds. Herds, which recently experienced an increase in bulk-tank milk antibody levels, were probably less likely to be misclassified. Moreover, the increased antibody levels in milk could be delayed compared to the time of introduction of new infection to the herd (Jordan et al., 2008). This could lead to false negative YQs for some

herds and even some low-prevalence herds being misclassified as test-negative altogether (Veling et al., 2002). Hence, misclassification could potentially bias our results. We therefore reanalysed the data with a more strict definition of new infection events – requiring two consecutive test-positive YQs instead of one in Dataset B and C. The final model results (data not shown) were very similar to those shown in Table 4, and the parameter estimates only became more extreme suggesting that the predictors may in fact have an even higher impact on the hazard than estimated using Dataset A. Based on this and the evaluation of the model fit investigations, we conclude that bias is not likely to be an issue of concern in this study. This led us to conclude that the predictors for new infection events identified in this study are important target points in a control programme for *S*. Dublin in cattle.

Suggestions for further studies

A study on time-dependency of predictors for recovery (or becoming test-negative) for *S*. Dublin is warranted to investigate the effect of the farmer experience groups compared to the general surveillance and control programme. Moreover, it would be useful to include predictors not readily available from register data, such as hygiene levels, management and housing facilities, feeding strategies etc. In an observational study of 84 *S.* Dublin test-positive dairy herds, Nielsen et al. (2012b) found that good calving management and hygiene, single pen calf housing with solid walls rather than bars, preventing cows from calving before being moved into the a designated calving pen, and good consistent colostrum feeding practices were associated of successful control of *S*. Dublin in the calf barn, but further studies on the effect of management factors are needed.

Conclusion

The conclusions and recommendations based on the results of this study can be summarized as follows: The incidence risk of *S*. Dublin decreased gradually during the surveillance period in Danish dairy herds and was reduced faster during the intensified control period from late 2007 and onwards. Herds with prior infections need to maintain a high level of internal and external biosecurity for at least 3 years after becoming test-negative to prevent recurrence. Furthermore, general recommendations for dairy herds wishing to protect their herds against *S*. Dublin include avoidance of purchase from test-positive herds and other external biosecurity measures. Large herds, herds with infected neighbours within a 5 km radius and herds with high somatic cell counts should, furthermore, focus on obtaining and maintaining a high level of internal biosecurity (e.g. hygiene and sectioning of groups of cattle).

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PAPER VIII

Time-to-event analysis of predictors for recovery from *Salmonella* Dublin infection in Danish dairy herds between 2002 and 2012

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Time-to-event analysis of predictors for recovery from *Salmonella* Dublin infection in Danish dairy herds between 2002 and 2012

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Abstract

Salmonella Dublin infections reduce gross margins and compromise animal health and welfare in dairy cattle herds. Despite on-going control efforts in several countries, the duration and risk factors of a persistent infection have been difficult to study due to a lack of suitable data. This study utilised the unique opportunity to extract systematically collected repeated bulk-tank milk antibody measurements from all the Danish dairy herds during a 10-year period to perform a time-to-event analysis of the factors that affect the duration of test-positivity and the hazards of recovery from *S*. Dublin at herd level.

Recovery was defined as a shift from test-positive to test-negative between two year-quarters followed by at least three more test-negative year-quarters. The average duration of infection was approximately 2 years. Predictors of recovery were tested in a multivariable Cox proportional hazard model allowing herds to recover from infection multiple times over the 10-year surveillance period. The model results were based on 36,429 observations with data on all the predictors, representing 3563 herds with a total of 3246 recoveries. Sixty-seven herds (2.4%) remained test-positive throughout the study period. The rest of the 317 herds that did not have any recoveries were censored, mainly due to a cessation of milk production.

Prior recovery from test-positivity turned out not to be a significant predictor of recovery in the model. The effect of the duration of infection on the conditional probability of recovery (i.e. the hazard) was time-dependent: early in the study period, long durations of infection were predictive of a low hazard of recovery. Later in the control programme, the effect of duration of infection was reduced indicating a desired effect of an intensified control programme. There was an increasing tendency towards longer durations and lower hazard of recovery with: (i) increasing herd sizes, (ii) increasing bulk-tank milk somatic cell counts, (iii) increasing local prevalence within a 5 km radius, (iv) organic farming and (v) recent purchase of cattle from test-positive herds. Participation in a voluntary paratuberculosis control programme reduced the duration of infection, and there were indications that recovery from *S*. Dublin infection was stimulated by a centrally organised and targeted control campaign. This is the first large-scale study that investigated duration of infection and predictors of recovery from *S*. Dublin in cattle herds over an extended period of time. The results provide useful knowledge for the design of control programmes for *S*. Dublin.

Introduction

Salmonella enterica subsp. *enterica* Dublin (*S*. Dublin) is a cause for concern in cattle industries across many countries (McDonough et al., 1999; Bergevoet et al., 2009; Carrique-Mas et al., 2010; Lewerin et al., 2011) because of the economic losses and welfare consequences such as high mortality, morbidity, abortions and a reduced milk yield (Richardson and Watson, 1971; Visser et al., 1997; Nielsen et al., 2010; 2012b). Furthermore, infrequent but serious human infections occur and are mainly attributed to the consumption of beef or unpasteurised milk products (Maguire et al., 1992; Helms et al., 2003).

Several studies indicate that *S*. Dublin has a tendency to persist in dairy herds due to the environmental survival of bacteria (Taylor and Burrows, 1971; Findlay, 1972) and the persistent infection in some cattle (House et al., 1993). Apart from the hygiene and management factors, persistency also depends on the herd size and the epidemic size of the initial infection episode or outbreak (Veling, 2004b; Nielsen et al., 2012). Veling (2004) found that close to one third of 49 dairy herds that experienced an outbreak of *S*. Dublin were still culture-positive 14 months after the initial outbreaks. However, few observational studies have attempted to estimate the duration of *S*. Dublin infection in dairy herds, most likely because it requires intensive sampling of many herds over many years (Boqvist and Vågsholm, 2005). Jordan et al. (2008) used 3 years' worth of Danish surveillance data and found that the duration of 'the true positive period' was an exponential distribution with a mean of 726 days. This corresponds to the time that a herd was infected and had antibody positive bulk-tank milk (BTM). To this, should be added up to 120 days for dissemination of the infection and consequent rise in antibody titres before the BTM became positive (Jordan et al., 2008). These estimates may be too low due to some uncertainty related to the short time period that the data was available.

In Denmark, a national surveillance programme for *S*. Dublin covering all of the cattle herds was initiated in 2002 (Anonymous, 2004, 2012). Dairy herds were classified into infection categories based on screening for BTM antibodies four times per year and the monitoring of cattle movements between herds with different test-statuses (Warnick et al., 2006). The information was stored in the Danish Cattle Database, and therefore provided a unique source of information for an investigation into the duration and the factors affecting the duration of *S*. Dublin in Danish dairy herds. In 2007, a national control campaign was initiated in which infected herds were encouraged to eradicate *S*. Dublin by the use of management and test-strategies (Nielsen and Rattenborg, 2011). The prevalence of test-positive dairy herds was reduced from approximately 26% in January 2002 to 8% in May 2012, with the last 8%-points of this reduction occurring from 2007 to 2011 during the control campaign. It was hypothesised that the duration of infection was reduced during the centrally organised control campaign from late in 2007 and onwards, and that this could be assessed by an analysis of the time-variance of the duration and the predictors in a survival analysis of the available surveillance and register data. Previous studies suggest that the relevant predictors to include in the analysis were herd size, local prevalence, purchase patterns and management factors which can be evaluated using indicator variables, such as organic vs. conventional farming and somatic cell counts when based on register data from a cattle database (Veling, 2004; Nielsen et al., 2007). The objectives of this study were: 1) to estimate the average duration of *S*. Dublin, 2) to evaluate the potential time-dependency of the duration of infection over the study period from April 2002 to March 2012, and 3) to investigate the predictors and the time-dependency of the effect of the predictors for recovery from an *S*. Dublin infection in dairy cattle herds.

Materials and methods

Sampling and categorisation of herds

Data from the Danish surveillance programme for *S*. Dublin in cattle herds were extracted from the Danish Cattle Database (DCD). In the programme, dairy herds have had BTM samples collected, on average, every three months since the beginning of 2002. We included data from January 2002 to end of 2011. The BTM was analysed for antibodies directed against *S*. Dublin lipopolysaccharide O-antigens using an in-house ELISA, resulting in a background corrected optical density value

(ODC%), which can be interpreted as a semi-quantitative indication of the level of antibodies in the sample (Nielsen and Ersbøll, 2005). In the surveillance programme, herds had their classification re-evaluated every time the new BTM ELISA results were available. However, due to the quarterly sampling scheme, and to facilitate a further analysis of these data, the herds were classified on a year-quarterly basis similar to the classification procedures used in the surveillance programme (Warnick et al., 2006). If the average of the last four BTM measurements were below 25 ODC% and the most recent BTM measurement was not >20 ODC% above the average of the previous three BTM measurements, the herd was classified as test-negative in that given quarter of the year (YQ). Otherwise the herd was classified as test-positive. If there were no BTM measurements in a given YQ, the herd was classified as test-positive as long as either the previous or the following YQ was test-positive. This classification method was evaluated by Warnick et al. (2006), who found that the herd sensitivity was 0.95 independent of prevalence, and that the herd specificity varied from 0.83 at an underlying prevalence of 50% to 0.98 at 2% infected herds.

The risk of persistent infection was investigated by time-to- event analysis, where the event was 'recovery from *S*. Dublin infection'. The herd status was set to missing if not enough consecutive YQs were available to potentially obtain a recovery status. YQs in which the herds had fewer than 10 cows were excluded from the analyses because this situation typically occurred when a herd went out of business, and the BTM test results were considered less accurate in that situation. Herds were considered non-infected (0) if they were test-negative for at least three consecutive test-negative YQs, and infected (1) if they were test-positive or if two test-positive YQs were separated by one or two test-negative YQs (i.e. for the patterns 1-0-1 and 1-0-0-1, the zeros would be considered as infected, because the herd was assumed to have the infection present even though the BTM antibody test did not pick it up during this period, i.e. false negative YQs). A change from test-positive to test-negative followed by at least two more test-negative YQs was considered a recovery. Gaps (not at risk of recovering) in the dataset occurred when herds were either considered non-infected according to the criteria, or the infection status of the herd was missing due to a lack of sufficient data.

Construction of the predictors from register data

Routinely collected data on the geographical location of herds, the movement of animals, the production practices and milk quality control data was extracted from the Danish Cattle Database from January 1st, 2002 to March 30th, 2012. Furthermore, BTM test results from 2001 were used to determine the herd test status at the beginning of the study period in 2002. The following variables were constructed for each YQ for further analysis:

Year quarter (YQ): YQ was based on the months January to March, April to June, July to September and October to December. The distribution number of dairy herds at risk of recovery and the probabilities of recovery for each YQ in the study period are shown in Fig. 1.

Season: Season followed the sampling periods in most of the study period equal to the months used to define the YQs.

Farming type: Information about registered organic farming in a given YQ was obtained from the Danish Plant Directorate and merged with data from the Danish Cattle Database. Most of the herds were either organic or conventional during the whole period.

PTB: Herds enrolled in a voluntary control programme for paratuberculosis that was initiated in Denmark in March 2006 were encouraged to optimise biosecurity procedures to prevent an introduction and spread of the infection. This might also affect the probability of clearing an *S*. Dublin infection. Therefore, this variable indicates whether herds at risk of recovery were recorded as enrolled in the paratuberculosis control programme in the given YQ.

Purchase: Based on movement data this variable indicated whether herds had purchased no animals at all, only animals from *S.* Dublin test-negative herds or if the purchases had included the purchase of animals from *S.* Dublin test-positive herds in the current or previous YQ. The *S.* Dublin test-status of the source herds on the date of the actual purchase was used to create this variable.

Herd size: The herd size was recorded as the total number of animals recorded on the premises in each YQ. Observations (YQs) in which the herds had fewer than 10 cows recorded were excluded from the analyses (1.8% of the observations in the dataset). This was done because the BTM antibody measurement in such YQs would not lead to accurate herd classifications and such YQs usually occurred toward the end of the production period for dairy herds going out of business. For the analysis the herd size was categorised into four levels equivalent to the four quartiles of the herd size distribution.

Prior recoveries: An indication of whether the herd had prior recoveries (and therefore also prior infections) recorded in the surveillance programme counting from the second YQ of 2002.

Local herd prevalence: The number of test-positive non-dairy and dairy herds in a 5 km radius divided by the total number of herds in that geographical area for each YQ.

Local cattle density: The number of cattle in all cattle herds in a 5 km radius for each YQ.

LogSCC: The logarithm of the average bulk-tank milk somatic cell count measured during the given YQ divided by 1000.

Duration (time at risk): The duration of infection, which is equal to the time at risk of recovery, was counted as the number of consecutive test-positive YQs including the current YQ.

Statistical analysis

Descriptive analyses were performed on all of the variables including the number of YQs and the number of herds represented within each level of the categorical variable. The term 'hazard' implies the probability of the event 'recovery' in a given YQ conditional on the herd having remained infected until the beginning of that YQ. Time-to-event analysis was performed using a multivariable Cox proportional hazard model taking into account the within-herd clustering effect of herd statuses over time and allowing for multiple failures (recovery events) to occur in the same herd. We used the 'Anderson-Gill model', and included a covariate to indicate whether the herd had recovered from *S*. Dublin infection before or during the study period to relax the assumption that of recurrence is assumed to be independent of previous events (Andersen and Gill, 1982; Wei and Glidden, 1997). The analysis was performed in STATA® IC/12 (StataCorpLP, College Station, Texas, USA).

Only YQs in which the herd could be considered at risk of having a recovery event (n= 38,024) including those in which they actually had a recovery event (n=3,351) were included in the analysis. YQs not included in the analysis were considered gaps, either due to uncertain herd test status in that YQ according to the criteria for being at risk, or because the herd was test-negative, and therefore not at risk of recovering. The dataset was split at each event. Ties in the data (i.e. multiple new recovery events across the dataset in the same YQ) were handled using Efron's method. Reasonable functional forms of continuous predictors were determined by the evaluation of lowess smoothed graphs of Martingale residuals for the original variable, and if necessary appropriate transformations of the original variable were performed as described by Dohoo et al. 2009.

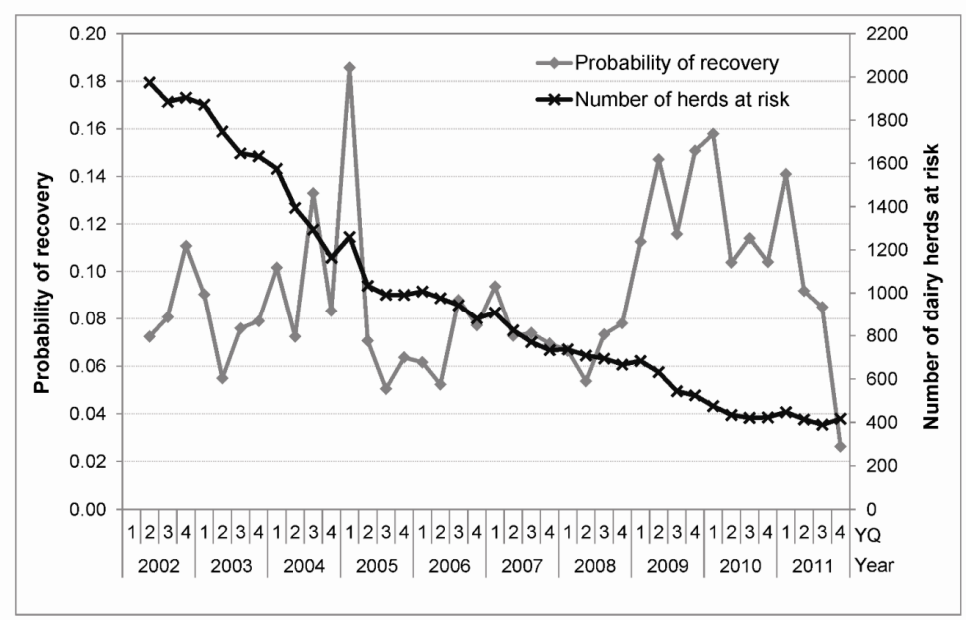

Figure 1 The grey line relates to the left y-axis and shows the probability of recovery from *S*. Dublin per year-quarter (YQ). The black line relates to the right y-axis and shows the number of dairy herds at risk of recovering from *S*. Dublin (i.e. having at least three consecutive test-positive year-quarters prior to the current) according to bulk-tank milk surveillance data from all of the Danish dairy herds from 2002 to 2012.

A stepwise backwards selection procedure was used to test the main effects of the predictors including all of the possible two-way interactions and evaluations of time-varying effects. Due to the size of the dataset, the main effects were included in the final model at a 1% significance level. The interactions were evaluated for both statistical significance and biological importance, meaning that statistically significant interactions with very low parameter estimates rendering the effect of the interaction essentially unimportant in the interpretation of the results were excluded from the final model after visual inspection of survival and hazard function plots with and without the given interaction in the model. The removed non-significant predictors were reintroduced to check for confounding. A change of more than 20% on any of the parameter estimates in the final model after the re-introduction of a predictor was considered as a confounding effect and the predictor would then stay in the model. The assumption of proportional hazards was evaluated by

a statistical test evaluation of Schoenfeld residuals for variables included in the final model to evaluate whether or not there was evidence that some hazard ratios were not constant over time (Dohoo et al., 2009). Time -varying components were included in the final model for predictors with hazards that were not constant over time. The best functional form of the time-varying component was evaluated by comparing the log likelihoods between the different models. For models with similar log likelihoods the simpler model was selected to ease the interpretation. The assumption of independent censoring was evaluated by sensitivity analysis comparing scenarios with changed positive and negative correlations between censoring and new infection events. Outliers were checked for by plots of deviance residuals vs. time and influential points by plots of score residuals vs. time.

Results

There were 3,577 herds that contributed with some time at risk of recovery during the study period (n=38,024 YQs). These herds had a total of 3,351 recovery events. In the raw data, the herds were coded as infected on average 9.9 YQs (median: 7 YQs) across the whole surveillance and control period. The overall mean number of recoveries per herd was 0.94, and there were between 0 and 5 recoveries per herd. A total of 67 herds remained infected or test-positive as long as they were active as dairy herds. The overall unadjusted probability of recovery per YQ was 8.8%. These numbers should be seen in relation to the total of 8,281 dairy herds that delivered milk to the dairy companies for at least two YQs during the study period, and 3,828 of these were test-positive for at least one YQ. Due to the rapid structural changes in the Danish cattle population towards fewer and larger dairy herds, there were only 3,828 active dairy herds left with more than 10 cows in the first YQ of 2012, and 396 of these (10.3%) were test-positive. The discrepancy between this prevalence and the prevalence reported in the national surveillance programme was mainly due to the fact that we removed herds with fewer than 10 cows from the dataset for analysis. One herd had four recoveries, three herds had three, 62 herds had two, and 454 herds had one recovery in the dataset prior to the last recovery event recorded in the study period. Table 1 shows the differences in probability of recovery between different levels of the categorical predictors. Descriptive statistics of all of the continuous variables and the discrete variable “YQs at risk” are provided in Table 2. Table 3 shows the descriptive statistics of the duration of infection in eight categories of herd size and farm type.

The functional forms of the continuous predictors were evaluated to be reasonable as linear effects. Table 4 shows the resulting model. The model results were based on 36,429 observations with data on all of the predictors, representing 3,563 herds with a total of 3,246 recoveries. The hazard of recovering from *S*. Dublin did not differ significantly between the seasons and was not associated with local cattle densities as long as local prevalence was in the model simultaneously. Predictors found to significantly increase the hazard of recovery (and thereby shortening the duration of infection) were: conventional farming type, smaller herd size, lower local prevalence, no purchase or purchase restricted to test-negative herds and lower bulk-tank milk somatic cell counts. There was a significant interaction between the local prevalence and the participation in the paratuberculosis control programme indicating that it was only under high prevalence conditions that participation in the paratuberculosis programme decreased the duration of infection as illustrated in the survival curves in Fig. 2. Furthermore, the effect of the local prevalence of *S*. Dublin varied with the duration of infection, and the effect of the duration was time-dependent. The resulting predicted smoothed hazard functions over the 10-year surveillance

period are illustrated in Fig. 3 for different combinations of predictors and assuming conditions representative of two different times (i.e. the third YQ of 2004 and the third YQ of 2009, respectively), and two different duration of infection.

Table 1 Descriptive statistics of the categorical predictors and the probability of recovery from *S.* Dublin for each level of the predictors based on register data from all Danish dairy herds with more than 10 cows between 2002 and 2012.

Predictors	Number of YQs at risk	Probability of recovery
Season		
January-March	9,340	10.4%
April-June	10,141	7.3%
July-September	9,581	8.7%
October-December	9,338	8.6%
Farming type		
Organic	5,003	6.9%
Conventional	33,397	9.0%
PTB		
Not enrolled in the PTB-programme	34,872	8.7%
Enrolled in the PTB-programme	3,528	9.1%
Purchase in the current and previous YQ		
No purchases	20,721	9.4%
From test-negative herds	11,119	10.0%
From test-positive herds	4,589	4.1%
Somatic cell count level in the current YQ		
Log(scc) ≤ 5.5	18,024	9.8%
5.5 > Log(scc) ≤ 5.7	9,294	8.3%
Log(scc) > 5.7	11,082	7.4%
Herd size levels		
>11-150	9,650	12.1%
>150-229	9,620	8.8%
>229-311	9,574	7.7%
>311	9,556	6.3%
One or more prior recoveries in the study period		
Yes	5,024	11.9%
No	33,376	8.3%

The predicted hazards of recovery were similar at both times for herds with duration of 2 YQs, but was markedly higher late in the programme when duration was long (20 YQ used as an example) (Fig. 2A and 2B) indicating that the effect of the duration of infection on the hazard of recovery was reduced over time. Fig. 4 illustrates the predicted smoothed hazard functions for herds with low hazards of recovering from *S*. Dublin due to a combination of high risk predictors, assuming

conditions similar to the third YQ of 2009. For this combination of predictors the hazards of recovery was even lower when assuming conditions similar to the beginning of the study period.

Table 2 Descriptive statistics of continuous variables tested in a survival analysis model of predictors for recovery from *S.* Dublin infection in Danish dairy herds between April 2002 and March 2012. The variables were collated per quarter of the year (YQ) in the study period.

Predictors	Min	Mean	Median	Std	Max
Duration (number of YQs at risk)	1	9.9	7	8.6	40
Herd size	11	259	229	185	3202
Local herd prevalence	0	0.15	0.14	0.09	0.5
Local cattle density[a]	27	5796	5746	2486	13790
LogSCC[b]	3.1	5.5	5.5	0.3	8.5

[a] Number of cattle in a 5 km radius around each study herd

[b] The natural logarithm of the bulk-tank milk somatic cell count in thousands averaged over the YQ

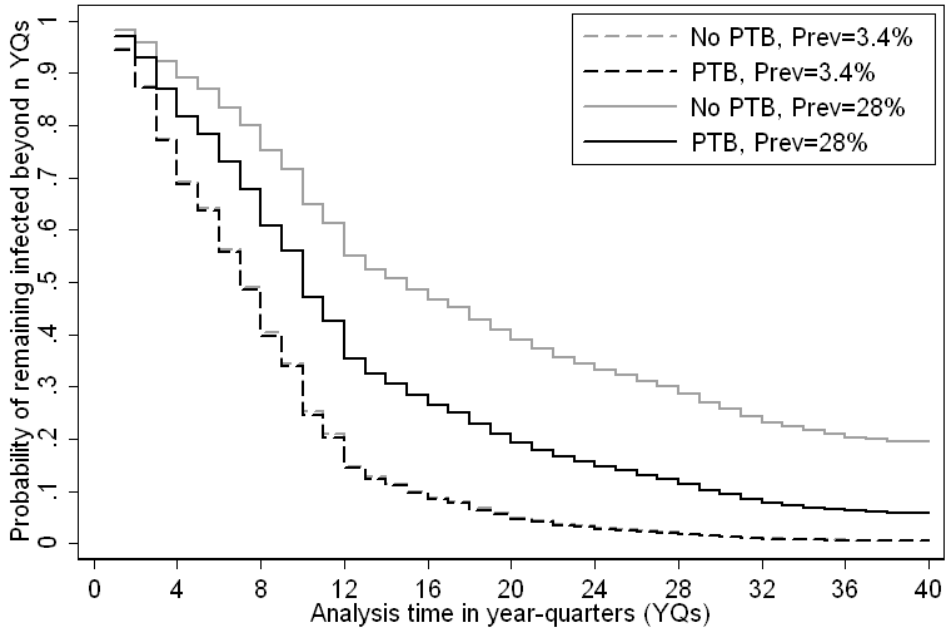

Figure 2 Predicted survival curves for recovery from *S*. Dublin infection in Danish dairy herds between 2002 and 2012 at low (3.4%) and high (28%) local prevalences. The black lines illustrate the predicted survival functions for herds participating in the voluntary paratuberculosis control programme (PTB), and the grey lines show predicted survival functions for herds not participating in the PTB.

The best model fit (i.e. the model with the log likelihood closest to zero) was obtained using an inverse analysis time to describe a time-varying effect of the duration. However, when displaying the results graphically using the actual analysis time in the interaction term between time and duration, the conclusions were very similar. Thus, to improve comprehensibility of the model results, the selected final model included a linear time-varying effect of duration. Prior recoveries were borderline significant in the model (P=0.05), however graphic displays of the hazard function with and without this predictor in the model showed that there was very little difference in the

hazard of recovery between herds with and without prior infections, so the effect was removed from the final model.

The assumption of independent censoring was evaluated to be reasonable by sensitivity analyses of correlations between censoring and new infection events, and we did not find influential outliers in the data.

Table 3 Descriptive statistics of durations of *S*. Dublin infections in eight categories of farming type and herd size for all Danish dairy herds under surveillance from 2002 and 2012.

Categories	Number of observations	Mean (median) duration in YQs [a]
Organic herds		
Herd size >10 to 150	693	7.6 (5)
Herd size >150 to 229	1531	9.4 (7)
Herd size >229 to 311	1537	10.0 (8)
Herd size >311	1242	14.2 (13)
Conventional herds		
Herd size >10 to 150	8957	7.0 (5)
Herd size >150 to 229	8089	8.5 (6)
Herd size >229 to 311	8037	10.6 (8)
Herd size >311	8314	13.3 (11)

[a] In all categories the minimum duration was 1 YQ and the maximum was 40 YQs, except in organic herds with herd size >150 to 229 which had a maximum duration of 39 YQs, and organic herds with herd size >10 to 150 which had a maximum duration of 33 YQs.

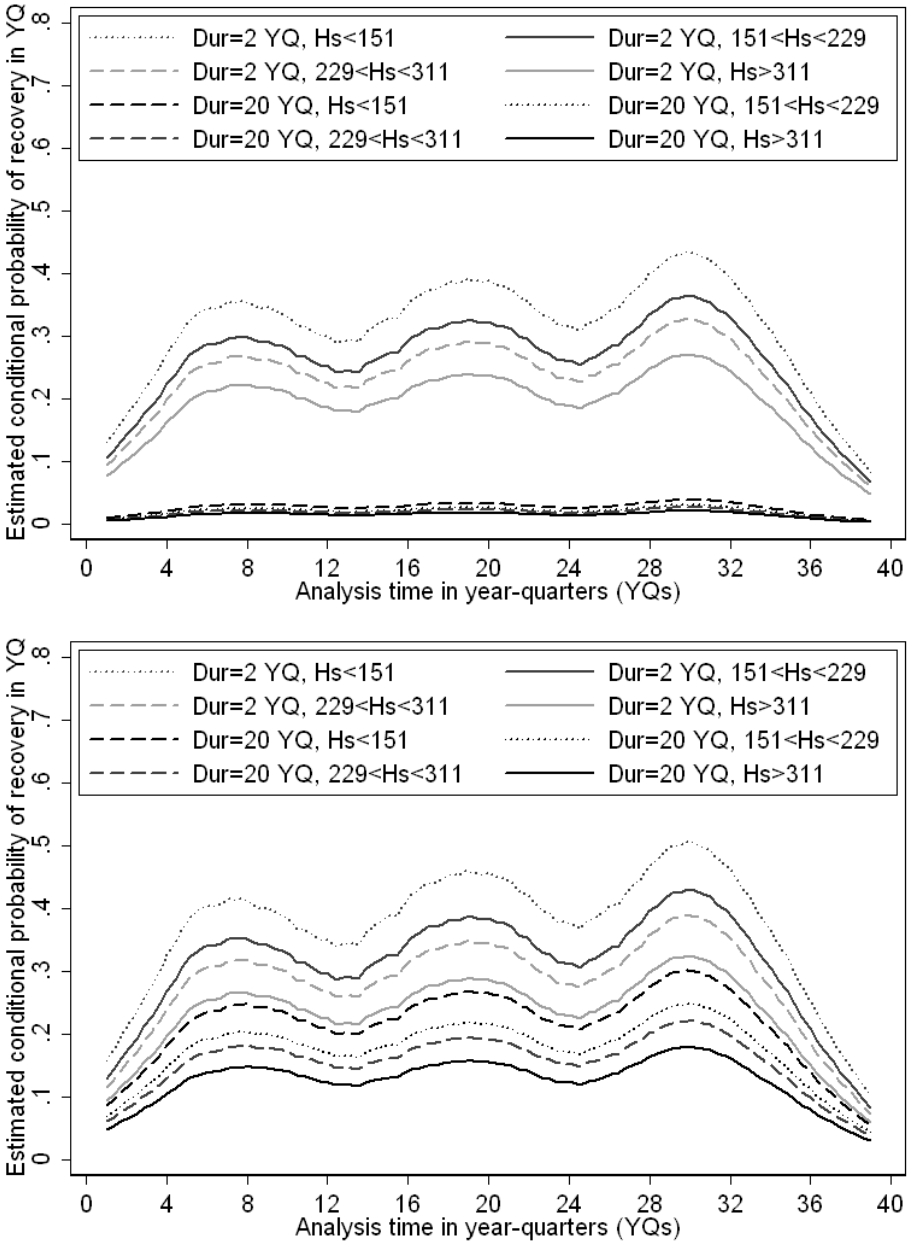

Figure 3 Smoothed hazard functions for recovery from *S.* Dublin infection in Danish dairy herds with four herd sizes and short (2 YQs) or long durations (20 YQs) at risk between 2002 and 2012 at a low local prevalence (3.4%). These herds were assumed to be conventional, to not have purchased any cattle and to participate in the voluntary paratuberculosis programme. The top graph shows the predicted hazards assuming conditions similar to those in the third YQ of 2004 where the surveillance programme had been in place for 2 years; and the bottom graph shows the predicted hazards assuming conditions similar to those in the third YQ of 2009, 2 years after the initiation of the intensified control programme.

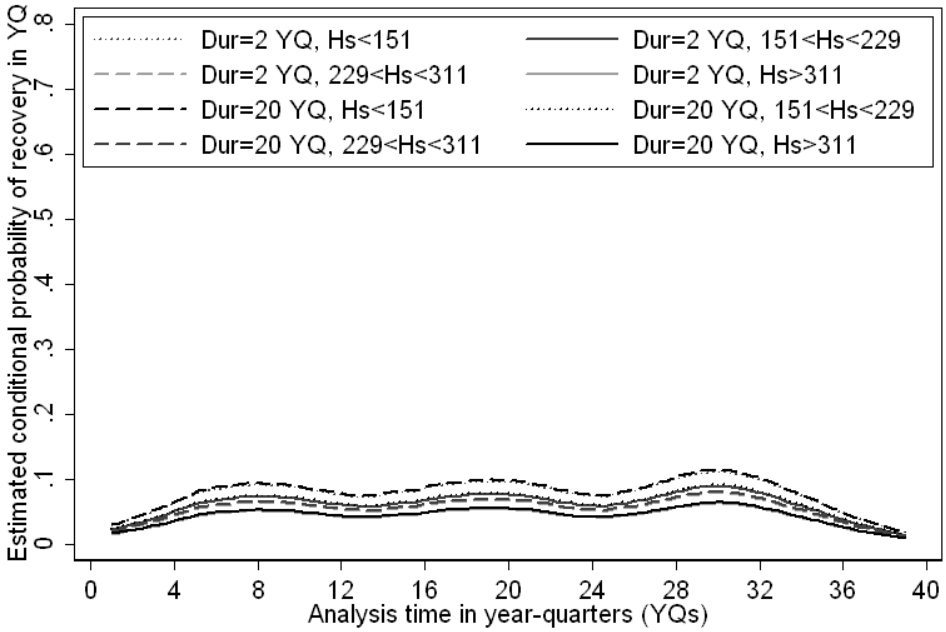

Figure 4 Predicted smoothed hazard functions for recovery from *S*. Dublin infection in Danish dairy herds with four herd sizes and short (2 YQs) or long (20 YQs) durations at risk between 2002 and 2012 at a high local prevalence (28%). These herds were assumed to be organic, to have purchased cattle from test-positive herds within the last 6 months, and not to be enrolled in the voluntary paratuberculosis programme. Furthermore, conditions similar to those in the third YQ of 2009 were assumed.

Discussion

This study investigated the duration and the predictors of recovering from *S*. Dublin infection in Danish dairy herds over a 10-year surveillance period through the use of a multivariable Cox proportional hazard model allowing for multiple recovery events to occur in the same herd and investigating the time-dependency of the predictors. To our knowledge this is the first large-scale study of the duration and risk factors associated with recovery from *S*. Dublin infections. The average duration of test-positivity in this study was 9.9 YQ corresponding to almost 2.5 years. Realising that it takes on average 60 days for antibodies to increase to measureable levels in BTM, and 180 days for antibodies to decline after a recovery from *S*. Dublin infection (Jordan et al., 2008), this suggests that the average duration across all infected herds during the study period of an infection was approximately 2 years.

Comparing to studies from other countries, a Swedish study found that 50% of the cattle herds were under restrictions for *Salmonella* (all isolated serotypes) for less than approximately 180 days. However, 4 out of 84 *S*. Dublin and 2 out of 21 *S*. Typhimurium infected cattle herds that were followed over time were difficult to clean up and were under restrictions with *Salmonella* diagnoses for more than 600 days after their first isolations (Boqvist and Vågsholm, 2005). The duration of infection in Swedish herds is difficult to compare to the Danish situation due to a very

strict enforcement of control actions aiming at the elimination of *Salmonella* from infected herds in Sweden.

Table 4 Final proportional hazards survival model for *S*. Dublin recovery in Danish dairy herds 2002-2012 with parameter estimates (β), standard error (S.E.), hazard ratios (HR), 95% confidence intervals for HRs and significance level (*P*).

Predictors	Estimate	S.E.	HR	(95%CI of HR)	*P*
Farming type					< 0.0001
Conventional	0	-	1	-	
Organic	-0.163	0.057	0.85	(0.76-0.95)	
Purchase					< 0.0001
No purchases	0	-	1	-	
From test-negative herds	0.106	0.039	1.11	(1.03-1.20)	
From test-positive herds	-0.548	0.080	0.58	(0.49-0.68)	
ParaTB control programme					0.648
Not participating	0	-	1	-	
Participating	-0.059	0.129	0.94	(0.73-1.21)	
Local herd prevalence[a]	-0.472	0.035	0.62	(0.58-0.67)	< 0.0001
ParaTB * Local herd prevalence[a]	0.219	0.086	1.25	(1.05-1.47)	0.01
Bulk-tank milk somatic cell count					< 0.0001
Log(scc) ≤ 5.5	0	-	1	-	
5.5 > Log(scc) ≤ 5.7	-0.177	0.045	0.84	(0.77-0.92)	
Log(scc) > 5.7	-0.308	0.046	0.73	(0.67-0.80)	
Herd size					< 0.0001
>11-150	0	-	1	-	
>150-229	-0.232	0.049	0.79	(0.72-0 .87)	
>229-311	-0.367	0.052	0.69	(0.63-0 .77)	
>311	-0.597	0.059	0.55	(0.49-0.62)	
Duration[b]	-0.204	0.011	0.82	(0.80-0.83)	< 0.0001
Duration[b] * local herd prevalence[a]	0.016	0.003	1.02	(1.01-1.02)	< 0.0001
Time varying effect					
Duration[b]* analysis time in YQs	0.005	0.0003	1.01	(1.00-1.01)	< 0.0001

[a] per 0.1 increase in prevalence, [b] per YQ increase of duration

Another study from the Netherlands suggested that approximately half of the dairy herds that experienced an outbreak of *S*. Dublin became persistently infected, meaning that there were still signs of new infections occurring after 14 months. The probability that the infection became persistent in the herd depended on how well transmission could be limited early in the outbreak (Veling, 2004). However, the herds in that study were not followed for more than 2 years, so it was not possible to systematically evaluate the expected duration and the factors affecting the duration of *S*. Dublin upon a new introduction of the infection to the cattle herds based on that study.

In our study, there was evidence that the control of *S*. Dublin was stimulated by centrally organised initiatives: the hazard of recovery from *S.* Dublin increased and remained high in 2002-2004 around the initiation of the surveillance programme (YQ 2 to YQ 11). Another wave of accelerated recoveries was observed from early 2006 to mid 2007 (YQ 16 to YQ 22). This was likely induced in early 2006 by a change in the surveillance classification programme, which led to a new wave of information material being distributed about *S*. Dublin and the changes in the legislation made it possible to reach the desirable 'Level 1' (test-negative) herd classification faster, when controlling the infection effectively. A third wave of increasing hazards of recovery started in YQ 25, approximately half a year after a regionally targeted control campaign was initiated involving direct contact to all test-positive herds and formation of local farmer experience groups. The local advisors were also engaged in the control campaign via a '*Salmonella*-consultant' from the Danish Cattle Federation

The positive effect continued throughout 2008 and 2009 up to around YQ 32, corresponding to the first YQ of 2010 (Figs. 3 and 4). After that the hazard of recovery appeared to decrease distinctly, which corresponds well to the stagnation in prevalence observed in the surveillance programme during 2010 and 2011 (unpublished data). In addition, the fact that the effect of duration of infection on the hazard of recovery waned over time, suggests that as the control programme developed, it encouraged farmers in herds with persistent infections to combat the infections more effectively than at the beginning of the surveillance programme. This may also have been caused by an increasing political pressure, information campaigns and peer pressure from other farmers, who were unwilling to accept the risk associated with having an infected neighbour farm. Certainly it appears that the voluntary control initiatives organised by the Danish Cattle Federation were successful in overcoming some of the problems reported with farmers' attitudes to biosecurity elsewhere (Heffernan et al., 2008). Lastly, the waning effect of duration may also be an indirect effect of the reduced local prevalence and further distance between herds over the study period due the structural changes in the cattle sector. This suggests that as prevalence decreases it should become easier to combat *S*. Dublin in previously high prevalence regions. This may be counter-acted by unresponsive farmer that have not yet done any efforts to control the infection in their herds (Kristensen and Jakobsen, 2011).

The fact that high bulk-tank milk somatic cell counts decreased the hazard of recovery and increased the duration of infection can most likely be explained by underlying management procedures, hygiene and occurrence of other diseases that favour the spread of the *S*. Dublin bacteria compared to a situation with low somatic cell counts, which is an indirect indicator of good hygiene and a good animal health status in the herd (Dohmen et al., 2010; Sant'anna and Paranhos da Costa, 2011).

Organic farming also decreased the hazard of recovery compared to conventional farming. This is probably related to regulations requiring longer contact between the dam and her new-born calf, and better opportunities for direct contact between neighbouring calves in the calf barn, which may also increase calf mortality in the organic herds (Nielsen et al., 2010). Furthermore, differences in treatment patterns may play a role (Fossler et al., 2005a, 2005b).

Participating in the paratuberculosis programme was associated with higher hazards of recovery and shorter durations than not participating, which is probably related to better internal biosecurity and restrictive purchase behaviours. This finding is not surprising, because the two infections have similar transmission patterns. However, this synergetic effect between two disease control programmes has not been described before and warrant further investigations. The specific internal biosecurity routines of relevance for persistence of an infection in cattle herds could not be investigated in this study, because it was based on register data from the Danish Cattle Database. However, such factors are important elements of the management challenges in the control of *S*. Dublin, including cleaning routines, housing facilities and barn sectioning, calving management, feeding practices, handling, administration of colostrum, control of other diseases and rodent control (Tablante and Lane, 1989; Hardman et al., 1991; Steinbach et al., 1997; Veling, 2004; Nielsen et al., 2012c).

Prior recovery from infection was not a significant risk factor in the model. This was surprising because one would expect that herds that had been infected before might have surviving bacteria in the environment and be exposed to a higher risk of a new introduction of *S*. Dublin to the livestock (Findlay, 1972; Nielsen and Dohoo, 2012). An explanation for this may be that we have other risk factors in the model that account for most of the risk of re-infections (e.g. purchase, herd size, local prevalence and predictors indicating level of hygiene in the herd) (Nielsen et al., 2007, 2012a; Nielsen and Dohoo, 2012). Furthermore, the strict recovery criteria used in this study probably prevented false negative YQs to be included in the analyses.

Because herd classification was based on the indirect measure of BTM antibody measurements over time, there might be concern that the criteria used to denote infection and recovery for each herd might have an effect on the results. To evaluate the effect of the herd classification criteria, two alternative scenarios were constructed in which the criteria for infection and recovery were altered. In the first alternative scenario we assumed that our recovery criteria were too strict in the original dataset, so for the pattern 1-0-1 the 0 was considered infected, but the two consecutive 0' in the pattern 1-0-0-1 were considered non-infected. This changed the number of recoveries from 3,351 recoveries in 3,590 herds in the original scenario to 3,632 in 3,580 herds in the alternative scenario, and the mean duration was changed from 9.9 YQs to 9.6 YQs. In the second alternative scenario the patterns 1-0-1, 1-0-0-1 and 1-0-0-0-1 considered the 0's infected. This changed the number of recoveries to 3,180 recoveries in 3,595 herds, and the mean duration changed to 10.1 YQs. However, in all three classification scenarios the median duration was 7 YQs. The final models included the same variables and had very similar parameter estimates as the original scenario. Hence, there appears to be no reason for concern about the used herd classification criteria in the overall estimation of duration and the interpretation of the predictors for persistence of *S*. Dublin in dairy herds. The duration of infection may still be over- or under-estimated, because of the indirect method of classifying herds as infected based on antibody levels in BTM. Overestimation might occur because of the antibody decay period after infection has

disappeared from the herd, and underestimation might occur, because BTM antibody measurements do not pick up all infection present in the herd (Warnick et al., 2006; Jordan et al., 2008).

The results were very similar when the model was run with the infection and recovery criteria defined in scenarios A1 and A2, so the model results were robust which increases confidence that the model reflects the predictors for recovery from *S*. Dublin infection and not just from test-positivity. In conclusion, several factors related to external and internal biosecurity, herd characteristics and farming practices determined the time to recovery from *S*. Dublin in dairy herds, and many of these practices appeared to be possible to affect through centrally organised control campaigns, field projects and legislation. The study results suggest that the effectiveness of control programmes for *S*. Dublin can be improved by ensuring centrally organised initiatives and field activities that positively encourage and motivate farmers and their local advisors to perform intervening control actions directed against the infection. According to the results of this study, the effect of each of the new initiatives at the national level lasted approximately two years.

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PAPER IX

Age-structured dynamic, stochastic and mechanistic simulation model of *Salmonella* Dublin infection within dairy herds

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Age-structured dynamic, stochastic and mechanistic simulation model of *Salmonella* Dublin infection within dairy herds

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Abstract

In the demand for a decision support tool to guide farmers wanting to control *Salmonella* Dublin (*S*. Dublin) in Danish dairy herds, we developed an age-structured stochastic, mechanistic and dynamic simulation model of *S*. Dublin in dairy herds, which incorporated six age groups (neonatal, preweaned calves, weaned calves, growing heifers, breeding heifers and cows) and five infection states (susceptible, acutely infected, carrier, super shedder and resistant). The model simulated population and infection dynamics over a period of 10 years in weekly time steps as: 1) population sizes of each of the six age-groups; 2) *S.* Dublin incidence and number of animals in each infection state; and 3) *S.* Dublin related morbidity and mortality in the acutely infected animals. The effects of introducing one infectious heifer on the risk of spread of *S*. Dublin within the herd and on the duration of infection were estimated through 1000 simulation iterations for 48 scenarios. The scenarios covered all combinations of three herd sizes (70, 200 and 400 cows), four hygiene levels indicating infectious contact parameters, and four herd susceptibility levels indicating different susceptibility parameters for the individual animals in each of the six age groups in the herd.

The hygiene level was highly influential on the probability that the infection spread within the herd, duration of infection and epidemic size. The herd susceptibility level was also influential, but not likely to provide sufficient prevention and control of infection on its own. Herd size did not affect the probability of infection spread upon exposure, but the larger the herd the more important were management and housing practices that improve hygiene and reduce susceptibility to shorten durations of infection in the herd and to increase the probability of extinction. In general, disease and mortality patterns followed epidemic waves in the herds. However, an interesting pattern was seen for acute infections and abortions in adult cattle after the first 2 years of infection in herds with poor hygiene and high susceptibility. Repeated infections in young stock lead to a high proportion of resistant adult cattle, which lead to a dampening effect on acute infections in adults and associated abortions. Sensitivity analyses of 24 alternative scenarios showed that a super shedder state was not essential to mimic the infection dynamics and persistence patterns known from field studies, but a persistent carrier state was required in the model to mimic real life *S*. Dublin infections.

Introduction

Salmonella enterica subsp. *enterica* serovar Dublin (*S.* Dublin) is a bacterium that is host-adapted to cattle and reduces animal health, welfare and production in infected cattle herds (Peters, 1985; Nielsen et al., 2010). Furthermore, it is a zoonosis leading to rare, but serious illness in humans (Maguire et al., 1992; Helms et al., 2003). In cattle the salmonella-bacteria are mainly shed in faeces and can therefore be transmitted from one animal to another by direct contact, e.g. calves licking each other or licking its own faecal-contaminated hair coat (Wray and Sojka, 1981). Other studies have shown that *S.* Dublin bacteria survive well in the environment. Therefore indirect transmission might

also occur from the barn environment, machinery or equipment, in water, on feeding areas or on pasture (Hardman et al., 1991; McLaren and Wray, 1991). Once established in a dairy herd, self-clearance of infection is not likely (Veling, 2004).

Vaccination is used as a tool to control *S.* Dublin in some countries (Richardson and Watson, 1971; House et al., 1993). However, the efficacy of vaccination is uncertain (Richardson and Watson, 1971) and vaccination strategies are rarely used to control zoonotic diseases in Denmark. Use of antibiotics has been associated with increased risk of *Salmonella*-excretion in heifers and cows, and has been suggested to produce latent *S*. Dublin carriers (Richardson and Watson, 1971; Warnick et al., 2003). Management procedures supported by testing strategies are necessary to achieve successful control of S. Dublin and eventually eradicate the infection from the herd (Jensen et al., 2004; Nielsen and Nielsen, 2011).

In 2002, a national surveillance and control campaign was initiated in Denmark. Farmers in test positive herds were encouraged to control the infection, and farmers in test negative herds were encouraged to protect their herds from introduction of *S.* Dublin (Nielsen and Rattenborg, 2011). Recommendations such as restrictive purchase policy, barn sectioning and hygiene measures to reduce infection have been communicated widely to all farmers as part of the Danish control programme for *S*. Dublin from 2003 and onwards.

It has been suggested that persistent infections at herd level cannot be controlled by only culling active carriers detected by bacteriological isolation (Veling, 2004). However, this and other hypotheses are difficult to test in field studies for several reasons. Farmers are reluctant to cull valuable heifers and cows, potentially confounding factors are difficult to control and intervention trials require a high number of herds that carry out a well-defined set of intervention procedures and a high number of control herds that do not intervene against the infection for an extended period (Nielsen and Nielsen, 2011). Furthermore, prevalence must be measured accurately before, during and after the intervention period, but a lack of sensitive and specific animal-level and herd-level *S*. Dublin diagnostic tests complicates measurement of success in such trials (Veling et al., 2000; Veling et al., 2002; Nielsen et al., 2004). Simulation modelling provides a cost-effective alternative to experimental field trials to improve understanding of within-herd infection dynamics of *S*. Dublin.

Existing models of *Salmonella* infection dynamics in cattle have mainly focused on estimating transmission parameters, and host and pathogen factors influencing probability of introduction and persistence of infection in the herd (Xiao et al., 2005; Chapagain et al., 2007; Nielsen et al., 2007a; Lanzas et al., 2008). Simulation models can also help determine which management procedures and test-strategies are preferable under different herd conditions (Kudahl et al., 2007). As illustrated by one previous model, pathogen-associated mortality and culling can change the herd dynamics (Xiao et al., 2005). Other complex, indirect effects of the infection are also likely to occur e.g. due to the tendency of this bacteria to cause abortions, periodic mortality and long-term infection in some individuals.

We developed an age-structured dynamic, stochastic and mechanistic Monte-Carlo simulation model of *S.* Dublin ("Dublin-Simherd") based on an existing model ("Simherd") that incorporates complex feedback mechanisms between e.g. reproduction and culling in addition to the main effects of *S*. Dublin in dairy herds (Østergaard et al., 2000; Kudahl et al., 2007). The aim was to use this model to investigate the effect of hygiene, management and herd size on the population and infection dynamics of *S*. Dublin in neonatal calves, pre-weaned calves, weaned calves, heifers and adult cows upon introduction of one infectious heifer to a dairy herd. The outcomes of interest were: i) the proportion of dairy herds that experienced spread of infection upon exposure to one infectious heifer, ii) time infection in herds that became infected, iii) epidemic size, i.e. number of animals affected (e.g. infected, clinically ill and dead) over a 10-year period, and iv) probability of extinction prior to 10 years from initial infection. Furthermore, we aimed to illustrate the effect of specific model assumptions on the results and conclusions.

Materials and methods

Overall structure of the Dublin-Simherd model

The model used in this study is essentially a new version of an existing model called "Simherd" which was developed over the last two decades and documented through 28 internationally published peer-reviewed articles. "Simherd" mimics real life Danish dairy herds of different sizes and incorporates the complex feedback mechanisms between reproduction, culling and feeding (Østergaard et al., 2000). The model is coded in Delphi 2006 in the software package Borland® Developer Studio (Borland®, Austin, Texas, US). It arranges animals in a virtual dairy herd as objects in computer memory. Each of the objects is assigned individual animal characteristics such as age, reproduction status, milk yield potential etc., which are updated through the progression of a dairy cow's life in weekly time-steps from birth to when it leaves the herd.

The model was originally developed to simulate and analyse production, reproduction, culling and health in dairy herds. Individual variation (e.g. in milk yield) at cow-level and discrete events (e.g. heat detection, conception, foetal death, sex and viability of the offspring, clinical disease, culling and death and commonly occurring diseases) are triggered stochastically using random numbers from relevant distributions. In 2005 the model was used for simulation of control of different mastitis pathogens (Østergaard et al., 2005). It has been further developed and a module was added to simulate the effects of paratuberculosis in dairy herds ("PTB-Simherd") (Kudahl et al., 2007). The Simherd-model has been developed over a period more than 20 years, and thus several versions have been developed, each one with verification and validation steps (Reeves et al., 2011) involving a comparison of the resulting outcomes of different simulations with what has been experienced in real-life dairy herds.

Before adding a *S.* Dublin-module to the existing version of the Simherd-model, 2140 parameters were used to design the underlying virtual dairy herd and 78 parameters were used to describe infection dynamics, test characteristics and decisions regarding paratuberculosis. This makes the model able to reflect real life dairy herds in which the farmer has the opportunity to adjust many practices and circumstances in the farm. Today, the Simherd-model is used commercially in Denmark for herd health consultancy, and it is presented here: www.simherd.com (accessed on the 18th of October

2011). It is beyond the scope of this manuscript to investigate the effect of the predefined parameters in the Simherd model. The Dublin-Simherd expansion incorporates all underlying dynamic population characteristics defined in the original PTB-Simherd model. In addition, it randomly allocates all animals in the simulated herd to one of five *S*. Dublin infection states at the beginning of weekly time steps according to probabilities that dependent on their infection state in the previous week (i.e. Markov Chain Monte Carlo simulation) and a set of 24 susceptibility parameters and 20 parameters determining probabilities of clinical disease, mortality and abortion as described below.

The model is mechanistic and dynamic in the sense that all individual animals and their current states are virtually stored in memory, and the animals are allowed to move between different production and biological states in each weekly time step over a 10-year period. The number of cattle in each state is then counted every week to represent the dynamics in the population. This ensures that all effects of disease and other events are associated with the individual animals and are therefore not counted more than once, when the model is going to be used to estimate the effects of *S*. Dublin infection and intervention against the infection in future studies. It is stochastic in the sense that probabilities determine if an animal experiences any of the discrete events at different stages in its life. For instance, a probability randomly determines whether a heifer becomes pregnant at the first AI, another probability at the second AI etc. In this study, we have repeated a set of scenarios in three standard start herds with herd sizes 70, 200 and 400 cows with the aim of being able to validate results by comparing with experiences from a Dutch field study (Weber et al., 2009) (70 cows), imitation of a typical Danish dairy herd (200 cows) and an assumed relevant future herd size (400 cows), respectively. All parameters, except those related to *S*. Dublin, were set to represent a typical Danish dairy herd with average production results and disease-levels, and they were kept constant in all scenarios. *S*. Dublin was introduced into the herd by simulating the purchase of one acutely infected (shedding) heifer without clinical symptoms 4 weeks before calving. This heifer had been infected for one week at purchase and due to stochasticity and specified management, it would in some iterations infect other heifers, in some iterations it would still be infectious after calving and thus potentially infect both calves in the calving area and cows later on, and in some iterations it would not infect other animals and the infection would die out. A model with underlying feedback mechanisms for culling and reproduction was chosen, because the infection dynamics interact with both these aspects through e.g. increased abortion and mortality risk. In future developments of the model decreased milk yield and test-and-cull strategies, which both affect culling decisions in the herd, will be incorporated.

Every weekly time step changing animal specific characteristics were updated, e.g. age, reproductive cycle stage, milk production. When the animal reached the relevant age and stage of production they were allocated to one of the six virtual age groups mimicking real life barn sectioning used by most dairy farmers when their cattle grow. The six age groups were: neonatal: 0 to7 days old, pre-weaned calves: 1 to 7 weeks old, weaned calves: 8 to 22 weeks old, growing heifers in common pens: 23 to 59 weeks old, breeding heifers: 60 weeks old to first parturition; and cows from first parturition until death or culling. The level of contact between animals within each barn section was specified to allow for modelling of different management strategies (e.g. improved hygiene or barn sectioning). Homogeneous mixing was assumed among animals within each of the six barn sections.

In the naïve herd, all animals were considered susceptible. Calves were always born susceptible in the model independent of the level of infection in the herd. However, susceptibility of cattle to *S*. Dublin depends on the age of the animal (Nazer and Osborne, 1977; Segall and Lindberg, 1991). Management influencing the physiological state of the animal also affects susceptibility to *Salmonella*-infections and could be an important source of variation in development of infection between individuals (Chambers and Lysons, 1979; Mattila et al., 1988; Morisse and Cotte, 1994; Wray and Davies, 2000). In the model, this was reflected by adjusting the susceptibility of animals within each age group based on an overall 'herd susceptibility level', probability of clinical disease and probability of dying (Table 1). The animal levels of susceptibility were estimated based on previous studies of the effect of different dosages of *S*. Dublin on clinical symptoms and shedding of bacteria for cattle of different ages (Nazer and Osborne, 1977; Segall and Lindberg, 1991). The "herd susceptibility level" ranged from 1 (best level) to 4 (poorest level) to indicate level of management (e.g. improved feeding of colostrum and milk, improved feeding of weaned animals, reduced density of cattle in the barn, clean and dry bedding, improved air quality and ad-lib access to water would lead to lower herd susceptibility). The default setting in the model was "herd susceptibility level 2", e.g. for the pre-weaned calf group this would respond to calf management that typically leads to healthy appearing calves, reasonably good feeding practices and feed quality, little draught in the calf barn and minimal stress on animals due to stocking density or lack of water and feeding space. More explanation of the susceptibility parameters is provided under the description of the simulation scenarios below.

Table 1 Susceptibility, morbidity and mortality parameters for each of six age groups defined in a simulation model for *S*. Dublin in Danish dairy herds.

	Susceptibility at the first exposure to *S*. Dublin					
Parameter	Neonatal	Pre-weaned	Weaned	Growing heifers	Breeding heifers	Adult
Herd susceptibility level						
Level 1	0.9	0.8	0.7	0.5	0.4	0.25
Level 2	0.95	0.9	0.8	0.6	0.5	0.3
Level 3	1	0.95	0.9	0.7	0.6	0.4
Level 4	1	1	1	0.8	0.7	0.5
Probability of clinical disease in acutely infected with *S*. Dublin	0.5	0.5	0.25	0.15	0.10	0.10
Probability of dying if clinically ill from *S.* Dublin if not treated	0.85	0.7	0.4	0.15	0.15	0.10
Probability of dying if clinically ill from *S.* Dublin if treated	0.75	0.5	0.20	0.10	0.10	0.05
Probability of abortion if acutely infected	NA	NA	NA	NA	0.15	0.15

Modelling the infection-recovery cycle

In contrast to the frequently used "susceptible-immune-recovered" model in which animals are considered *en masse*, this model keeps track of every "object" (individual animal), which is present in one of six age-groups and one of five infection states in each weekly time step. At each time step all animals get exposed to two sources of infection: 1) from infectious animals in the same barn section, and 2) from background environmental infection of the whole herd. The last source depends on the prevalence of infectious animals in the whole herd.

Figure 1 illustrates the modelled infection states animals could go through upon becoming infected with *S*. Dublin. The probability of becoming infected in the relevant barn section was a function of exposure and susceptibility of the exposed animal. This function was modelled according to the Reed-Frost epidemic model given by Eq. (1), which provided the probability of becoming acutely infected (A) in the current barn section for each object in each week time step:

$$P_A\,(barn\ section) = 1 - (1 - (\,k_b * s)/N_b)^{\,I_b} \quad (1)$$

where k_b is the number of effective contacts between the susceptible animal and other animals in the barn section per time step, s is the susceptibility of the animal as given by Table 1, N_b is the number of contactable animals in the barn section and I_b is the number of infectious contactable animals in the barn section (Abbey, 1952). The parameter I_b was calculated as total number of acutely infected, super shedders and carriers in every barn section for each weekly time step. Studies have suggested that carriers shed approximately 100 times fewer bacteria in faeces than acutely infected animals and that shedding is intermittent (Sojka et al., 1974; Mizuno et al., 2008; Nielsen et al., 2011). Therefore, in the model carriers were considered 100 times less infectious than acutely infected and super shedders. The effect of this was modelled by dividing the number carriers by 100. Super shedders were considered to be as infectious as acutely infected, but the duration of infectiousness was longer for super shedders than for acutely infected as described below.

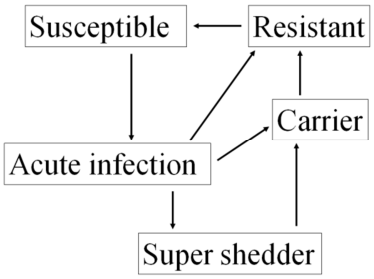

Figure 1 *S.* Dublin infection states included in the Dublin-Simherd simulation model. In the model each state was associated with a distribution of durations that animals spend in the different states and probabilities of changing to another infection state in the following week. Pathogen associated morbidity and mortality was included as probability distributions in the acute infection state. Only a small proportion of cattle that became acutely infected moved on to become super shedders or carriers. This progression

was determined by stochastic processes in the simulations, and probabilities were assigned according to previously collected field data and literature.

The number of effective contacts, kb, was estimated using both information about the dose-response relationship and transmission patterns from previous studies (De Jong, 1965; Taylor and Burrows, 1971; Taylor, 1973; Nielsen et al., 2007a), and the choices of kb, which can be found in Table 2, were evaluated by comparing the simulated number of infected animals and durations of herd infections with empirical knowledge from field studies from Danish and Dutch dairy herds (Nielsen, 2003b; Veling, 2004b; van Schaik et al., 2007; Jordan et al., 2008; Nielsen and Nielsen, 2011).The probability of background environmental infection that each animal was exposed to in every time step was proportional to the total number of infectious animals in the herd (I_e) and specified the probability of infection related to vehicle borne transmission of bacteria (e.g. via boots, tools, machinery, rodents) or direct contact between animals from other barn sections, e.g. through bars instead of solid walls between barn sections) as specified by Eq. (2).

$$P_A(environment) = 1 - (1 - (k_e * s)/N_e)^{I_e} \quad (2)$$

where the constant k_e was specified according to level of general internal biosecurity (denoted "hygiene level") in the herd (Table 2), N_e was the total number of animals in the herd, s was the susceptibility of the individual animal. The constant, k_e, used to model the probability of infection coming from the background (environmental infection) in Eq. 2 was obtained through an iterative calibration exercise in which simulations were performed and outcomes inspected until outcomes were obtained that indicated model behaviour to be plausible given the existing knowledge of the system and field outbreaks of *S*. Dublin.

Table 2 Model parameters used to specify the contact parameter k_b (number of effective contacts per week per animal in the age groups) and the contact parameter k_e for the background environmental infection to illustrate four different hygiene levels in a simulation model for *S*. Dublin in Danish dairy herds

Hygiene level	Neonatal calves k_b	Pre-weaned calves k_b	Weaned calves k_b	Growing heifers k_b	Breeding heifers k_b	Cows k_b	Background environment k_e
Good	0	0	1	1	2	2	0.02
Average	1	1	2	2	3	3	0.05
Poor	2	2	3	3	4	4	0.10
Very poor	3	3	4	4	5	5	0.15

Time spent in the acute infection state was modelled by sampling randomly from the distribution of durations in acutely infected animals illustrated in Fig. 2 at each weekly time step. This distribution was derived from published experimental and observational field study results (Robertsson, 1984; Nielsen et al., 2007a). Furthermore, animals in the acutely infected state were assigned probabilities of becoming clinically ill or dying from the infection for each of the six age-groups (Table 1). Those that

became clinically ill were considered ill for the whole period they were in the acute infection state. The risk of dying from *Salmonella*-induced clinical disease depended on whether the animal was treated or not, reflecting the farmer's ability to detect the symptoms and the threshold for applying treatment. Clinical disease, mortality and abortions were considered effects of *S*. Dublin rather than actual infection states and are therefore modelled as part of the acute infection state in the Dublin-Simherd infection-recovery cycle as described in section 2.3.

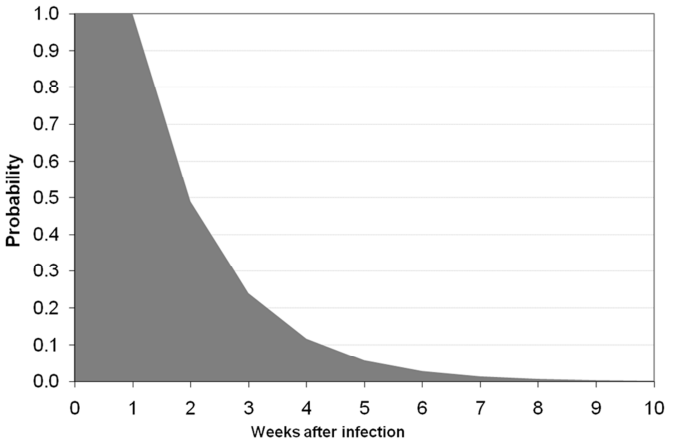

Figure 2 Distribution used to determine time spent in the acutely infected state before moving into either of the super shedder, carrier or resistant states in the Dublin-Simherd model

The probability of becoming carrier and super shedder were estimated from published and unpublished experimental and field studies. Hence, 18% of those that had been clinically ill in the acute stage were assumed to become carrier and 27% were assumed to become super shedder based on results reported by Robertsson et al. (1984) and Steinbach et al. (1997). Similar probabilities have been observed in an unpublished Danish field study. Animals that had not been clinically ill in the acute state were assigned 0.5% probability of becoming super shedder and 1.5% of becoming carrier after the acute state based on two published field studies of endemically infected dairy herds (House et al., 1993; Nielsen et al., 2007a). Finally, they could move directly to being resistant if not assigned to the carrier or super shedder states. Super shedders were considered infectious for an extended period of time determined by the distribution shown in Fig. 3. Calves below the age of one year were modelled as super shedder for at least 9 weeks, if they went into that state, whereas heifers and cows were modelled to be super shedders for at least 3 weeks. The difference is based on field studies – both published and unpublished - showing that calves tend to be infected for longer periods of time than older cattle (Nielsen et al., 2007a). Animals leaving the super shedder state moved to become carriers. The duration in the carrier state was determined by a two-phased distribution starting with a 36 week period in which all animals remained carriers followed by an exponentially decaying probability of remaining carrier up to 3 years after entering this state as illustrated in Fig. 3. This distribution was based on previous studies (Robertsson, 1984; House et al., 1993; Steinbach et al., 1997; Nielsen et al., 2007a).

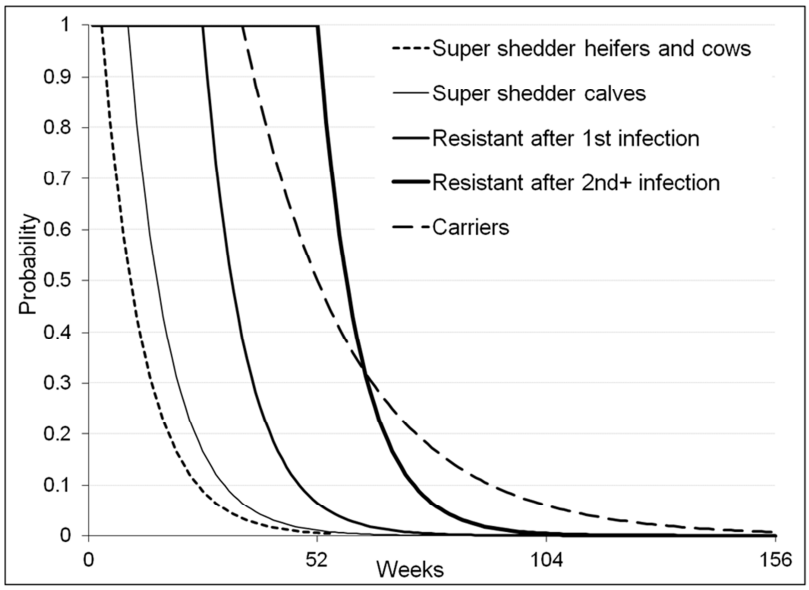

Figure 3 Modelled distributions of time spent in each of the super shedder, carrier and resistant states before moving into the next state in the Dublin-Simherd model.

The resistant period was modelled by the distribution illustrated in Fig. 3. Facing a lack of information about the duration of the resistant period in the literature we derived this distribution through discussions with experts from five different Danish institutions, who are experienced with *Salmonella* pathogenesis and infection dynamics. In addition to management factors and age, previous exposure to *S*. Dublin reduces the susceptibility of cattle (Steinbach et al., 1996). This was modelled by doubling the duration of the resistant period every time an animal became infected after having gone through the whole infection cycle. In practice this meant that during a normal life span of a cow (6-7 years), she could not become infected more than three times and this was specified in the model.

Modelling S. Dublin related morbidity and mortality

Table 1 provides the probabilities used to determine stochastically whether an animal became clinically ill together with probabilities of dying if treated and if not treated when becoming clinically ill in the model. These probabilities were estimated from literature on experimental and observational field studies of *S.* Dublin in cattle (Richardson and Watson, 1971; Taylor, 1973; Wray and Sojka, 1981; Smith et al., 1989; Wray et al., 1989; Segall and Lindberg, 1991; Smith et al., 1993; Silva et al., 2008; Carrique-Mas et al., 2010) combined with experience from unpublished experimental and observational field studies and expert opinion obtained through two discussion meetings with focus groups during the model development stages. No studies can provide the parameters directly, so they had to be estimated from comparison of the studies on different age-groups and using different dosages of *Salmonella*. It was evident from the studies that the older the cattle the higher the dosage required to make the animals clinically ill and die from the infection. It was assumed that different age

groups were exposed to similar dosages at each infectious contact, so it was mainly the susceptibility of the animal that determined whether an exposure led to infection.

Definition of introduction of infection and simulation settings

Farmers would be unlikely to purchase a grown animal with clinical symptoms of salmonellosis. Therefore, introduction of infection into the herd was modelled by purchase of one asymptomatic, acutely infected heifer. The model was used to simulate a 10-year period after introduction of *S*. Dublin to study infection dynamics in each age group. The outcomes of single simulations were discussed with experts to evaluate whether model parameters and outcomes of simulations seemed to be reasonable, relative to empirical knowledge of *S*. Dublin infections in cattle herds. To reduce the number of effects to take into account during the modelling procedure, we assumed that only one infectious heifer was purchased during the 10-year period and that the herd was not exposed to other sources of *S*. Dublin infection during the same period.

After the initial calibrations described in section 2.2, 1000 iterations were run for each of 48 scenarios representing all possible combinations of three herd sizes (i.e. 70, 200 and 400 cows), four levels of herd susceptibility (as shown in Table 1), and four levels of hygiene in the herds (as shown in Table 2). Good hygiene level implies minimal visible manure due to daily removal of manure from barn environment or manure covered with clean, dry bedding material daily. Average hygiene level implies some visible manure in the environment, clean and dry bedding, removal or proper cover-up of manure. Poor hygiene level implies build up of manure, dirty bedding, infrequent removal of manure, and very poor hygiene level implies extensive amounts of manure visible in the environment, dirty and wet bedding and rare removal of manure from the pens and barn environment in general. It is not possible, however, to validate how many effective contacts occur under these circumstances in real life. Therefore, the model illustrates the effect of relative changes in infection dynamics due to changes in hygiene procedures and housing types.

Sensitivity analysis of probabilities for becoming carriers and super shedders

Due to much uncertainty in the literature regarding the probability of becoming a persistently infected animal (i.e. latent carriers that can shed bacteria intermittently or super shedders that shed bacteria more or less continuously) and uncertainty about the infectiousness of such persistently infected animals, 24 simulation scenarios with changed combinations of probabilities of becoming carriers and without super shedders for different herd sizes, hygiene and herd susceptibility levels were performed and compared to the results from the original 48 scenarios. First, eight scenarios without carriers and super shedders were simulated (“No C and no Su” in Table 5). In practice this was done by setting the probability of becoming carrier or super shedder to 0%. Then, eight scenarios with carriers, but without super shedders were simulated (“C* and no Su”). In these scenarios it was assumed that the same overall proportion of animals would become persistently infected as in the original 48 scenarios (i.e. 18% carriers +27% super shedders). They would not become super shedders, but instead 45% (18%+27%) of the clinically ill acutely infected animals would become carriers.

Results

An example of the infection and population dynamics in the weekly time steps over a 10 year period is shown in Fig. 4 for the growing heifers (6-12 months old) in one of the 1000 iterations from the default scenario with 200 cows, average hygiene level and herd susceptibility level 2. The results of the simulations on three of the outcomes of interest (i.e. spread of infection, duration and probability of extinction) from the 48 scenarios are summarised in Table 3. These cover all the combinations of hygiene and herd susceptibility levels although some of the combinations are not likely to be seen in real life, e.g. very poor management leading to high susceptibility would rarely be combined with good hygiene and vice versa. However, they are all illustrated here to separate the effect of the number of contacts that might lead to infections and the susceptibility of the not yet infected animals, because these represent different control options.

Effects of herd size, hygiene and herd susceptibility on the probability of spread of S. Dublin infection

The probability that *S*. Dublin will spread in an exposed herd was measured as the number of herds with a 10-year total of more than one infected animal divided by the 1000 simulated herds. Overall, more than 60% of herds that were exposed to an infectious heifer had within-herd spread of infection (Table 3). The probability that *S*. Dublin would spread upon exposure 0.16 to 0.32 higher, when the hygiene level changed from good to very poor, depending on herd size and herd susceptibility level. The herd susceptibility level also had an effect, with 0.06 to 0.20 higher probability of experiencing spread of *S*. Dublin with very poor management implying high susceptibility compared to good management implying low herd susceptibility. Herd size did not have an effect on the probability that *S*. Dublin spread within the herd upon exposure (Table 3).

Effects of herd size and herd susceptibility on duration of infection and on probability of extinction in infected herds

Each iteration lead to different courses of infection in the herd. To illustrate how the infection could fluctuate in the barn sections an example of the outcome from one iteration out of 1000 of the default scenario is illustrated in Fig. 4 for the growing heifers. The infection came into this age group in week 7 in this iteration. In the figure, the number of animals in each of the infection states is stacked on top of each other for each weekly time step, so that the top line of the graph illustrates the total number of growing heifers in the group over time. This herd had a course of infection with multiple epidemics and the duration of infection in the whole herd was 295 weeks (5.7 years). This was typical for the default scenario in which most herds had several peaks of infection occurring in different barn sections, before the infection went extinct on average after 387 weeks (7.4 years) (median 489 weeks, 9.4 years).

To summarise the outcomes of all iterations that lead to infection in the default scenario, the mean number of infected animals in every weekly time step in each of the six age groups is displayed in Fig. 5. The much lower average numbers of infected animals among calves are mainly due to these groups being much smaller in the simulated herds than the older age-groups.

Table 3 Model predicted outcomes for probability of *S*. Dublin spread, duration of infection, proportion of herds with persistent infection after 1 year, and probability of extinction before 5 and 10 years at different basic settings in a simulation model for *S*. Dublin in Danish dairy herds. The estimates were derived from 1000 iterations of 10-year periods following introduction of one heifer with asymptomatic acute *S*. Dublin-infection one month prior to parturition.

	Hygiene level*	Herd susceptibility level**	Probability of *S.* Dublin spread	Mean herd infection durationin weeks	5^{th}–95^{th} percentiles of duration****	Proportion of herds still infected after 1 year****	Probability of extinction before 5 years****	Probability of extinction before 10 years****
Small herd (70 cows)								
	A	1	0.60	51	3-131	0.48	1.00	1.00
	A	2	0.69	65	4-149	0.59	1.00	1.00
	A	3	0.77	87	5-195	0.74	0.99	1.00
	A	4	0.80	99	10-225	0.77	0.97	1.00
	B	1	0.77	89	6-209	0.73	0.99	1.00
	B	2	0.83	114	11-257	0.82	0.95	1.00
	B	3	0.88	145	13-326	0.89	0.90	0.99
	B	4	0.92	174	23-397	0.92	0.80	0.98
	C	1	0.85	151	13-356	0.90	0.88	0.99
	C	2	0.89	174	42-404	0.93	0.81	0.98
	C	3	0.95	220	57-520	0.95	0.68	0.94
	C	4	0.96	246	72-520	0.98	0.63	0.90
	D	1	0.92	204	61-485	0.96	0.74	0.96
	D	2	0.95	234	71-520	0.96	0.64	0.92
	D	3	0.97	272	88-520	0.99	0.55	0.85
	D	4	0.98	300	98-520	0.99	0.48	0.80
Medium sized herd (200 cows)								
	A	1	0.63	94	4–199	0.75	0.99	1.00
	A	2	0.72	144	6–299	0.91	0.91	1.00
	A	3	0.78	217	64-500	0.96	0.69	0.96
	A	4	0.83	275	86-520	0.98	0.51	0.89
	B	1	0.81	290	66–520	0.96	0.50	0.81
***	B	2	0.84	387	125–520	0.98	0.25	0.58
	B	3	0.90	436	160–520	0.99	0.14	0.39
	B	4	0.93	462	189–520	0.99	0.10	0.28
	C	1	0.87	452	177–520	0.99	0.11	0.33
	C	2	0.92	473	226–520	1.00	0.07	0.22
	C	3	0.95	495	283–520	1.00	0.04	0.13
	C	4	0.97	503	352–520	1.00	0.03	0.08
	D	1	0.92	485	248–520	1.00	0.06	0.17
	D	2	0.95	500	313–520	1.00	0.03	0.10
	D	3	0.98	510	496–520	1.00	0.02	0.05
	D	4	0.98	516	520–520	1.00	0.00	0.03

(Table 3 continued)

Large herd (400 cows)							
A	1	0.63	148	4-353	0.80	0.88	0.99
A	2	0.70	257	5-520	0.90	0.58	0.85
A	3	0.74	403	68-520	0.95	0.22	0.48
A	4	0.84	466	164-520	0.97	0.10	0.24
B	1	0.78	476	104-520	0.96	0.08	0.15
B	2	0.83	506	520-520	0.99	0.02	0.05
B	3	0.90	515	520-520	1.00	0.01	0.02
B	4	0.92	518	520-520	1.00	0.00	0.01
C	1	0.87	514	520-520	0.99	0.01	0.02
C	2	0.92	518	520-520	1.00	0.00	0.01
C	3	0.96	519	520-520	1.00	0.00	0.00
C	4	0.97	520	520-520	1.00	0.00	0.00
D	1	0.93	517	520-520	1.00	0.00	0.01
D	2	0.96	520	520-520	1.00	0.00	0.00
D	3	0.98	520	520-520	1.00	0.00	0.00
D	4	0.99	520	520-520	1.00	0.00	0.00

* The hygiene level affects the number of contacts (k) in the model at shown in Table 2: A=Good, B= Average, C=Poor, D= Very poor.

** The herd susceptibility level affects the susceptibility of the animals in the age groups as shown in Table 1

*** Default scenario.

**** Estimated for those of the 1000 herds (iterations) where spread of *S*. Dublin was observed.

This does not necessarily mean that there were more clinically ill animals among the heifers and cows than among calves, because most of the infected animals in the older groups were asymptomatically infected. Whereas each iteration lead to different dynamics within the herd, the summary graphs in Fig. 5 illustrate that multi-epidemic patterns were common with a high peak epidemic few weeks after introduction of *S*. Dublin in all age-groups followed by approximately yearly peaks in the following years. In particular, the calf groups experienced repeated epidemics whereas the adult heifers and cows appeared to reach a more stable, declining level of infection after the first two years.

The duration of infection and the probability of extinction in infected herds was strongly affected by herd size, hygiene level and herd susceptibility. The larger the herd, the more important it was to have good hygiene level and low herd susceptibility to prevent *S*. Dublin from spreading and thereby reduce the duration and increase the probability of extinction. For herds with good hygiene level and herd susceptibility level 1, the maximum duration was 200 weeks for small sized herds, 352 weeks for medium sized herds and 520 weeks in large herds, and the probability of extinction before 5 years after introduction was between 0.88 and 1. In contrast, the probability of extinction before 5 years dropped to <0.11 in medium sized herd with poor and very poor hygiene. In large herds extinction was <0.15 within 10 years, if the hygiene level was not good.

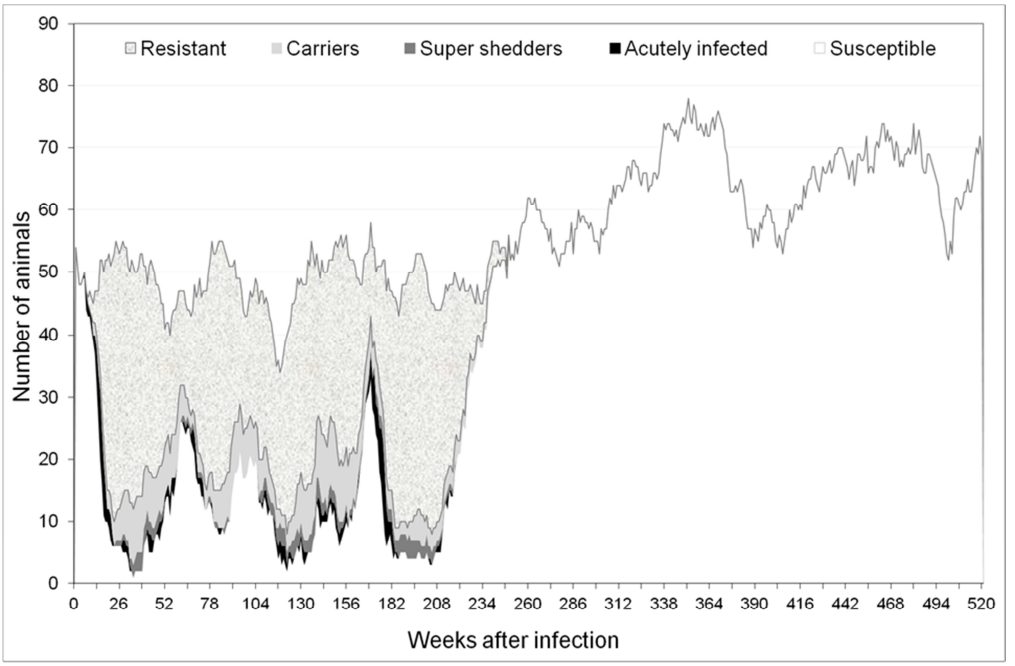

Figure 4 An example of the infection dynamics over a 10-year period in one of the six age groups of cattle (growing heifers 23 to 59 weeks old) in a simulated Danish dairy herd with 200 cows, herd susceptibility level 2 and average hygiene level (default scenario). The infection came into this age group in week 7. This herd had several epidemics of *S*. Dublin with a total of 131 diseased and 39 dead animals due to *S*. Dublin over the 5.7-year period that this herd was infected.

Effects of herd size, hygiene and herd susceptibility on epidemic size, morbidity, mortality and abortions in infected herds

As a consequence of the varying infection courses, the numbers of sick and dead cattle, and the number of abortions varied substantially between the infected herds. The numbers in Table 4 can only be compared within each herd size, but clearly the median number of sick and dead animals increased with poorer hygiene and increasing herd susceptibility independent of herd size indicating the importance of exposure to the bacteria and management that affects the susceptibility of the animals. The absolute numbers should be interpreted in the light of the different durations for each scenario (Table 3), and besides, the results in Table 4 are based only on those herds (iterations) where *S*. Dublin did spread within the herd.

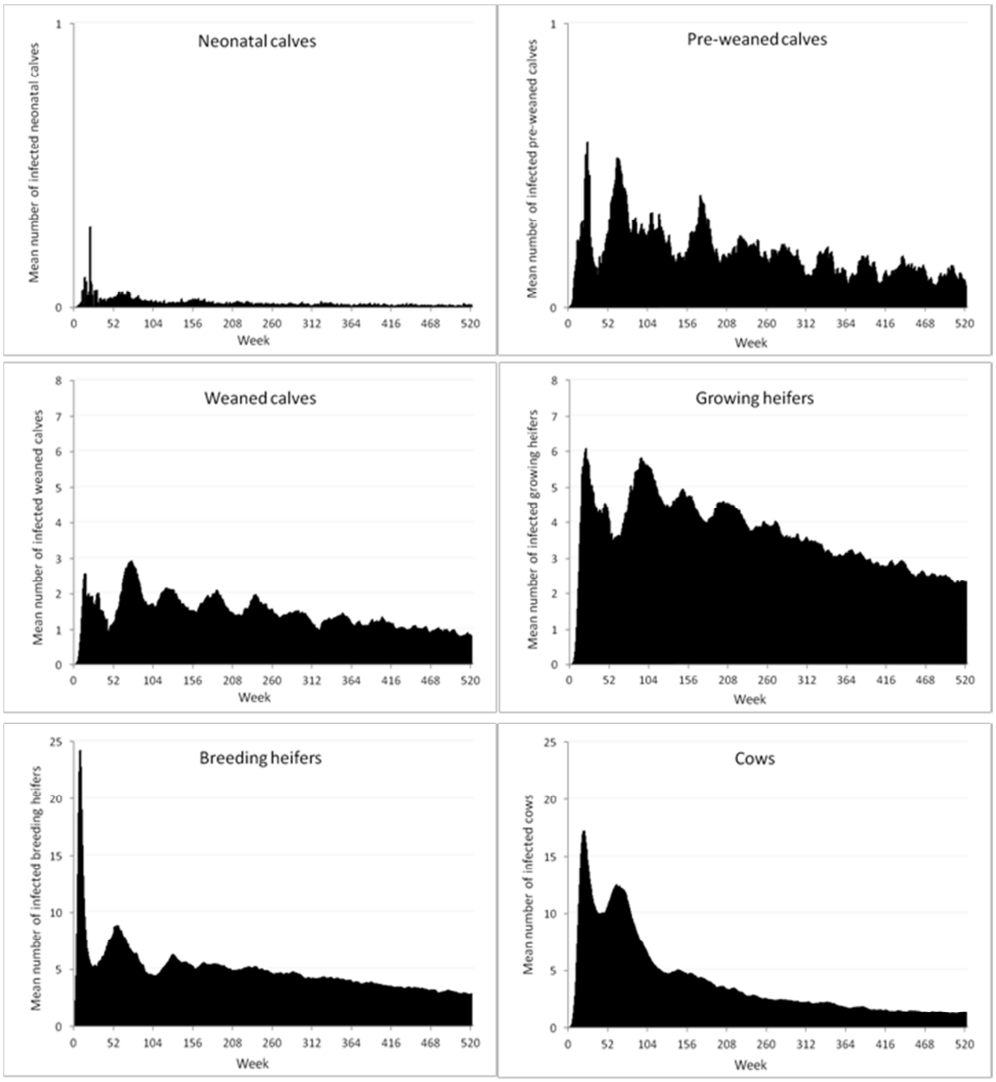

Figure 5 Mean number of infected animals during each week of the simulated 10-year period in the six age-groups in 835 out of 1000 iterations in which spread of infection occurred in the default scenario of the Dublin-Simherd model. Neonatal and pre-weaned calves are displayed at the top, growing calves in the middle, and adult heifers and cows at the bottom. (Note that the scales of the y-axes differ between age-groups.

Table 4 Model predicted outcomes of epidemic sizes (i.e. number of infected animals, number of clinically ill, number of dead animals and number of abortions) during the infected period in infected herds at different basic settings in a simulation model for *S*. Dublin in Danish dairy herds. The estimates were derived from 1000 iterations of 10-year periods following introduction of one heifer with asymptomatic acute *S*. Dublin-infection one month prior to parturition. The estimates in this table are from those of the 1000 herds (iterations) where spread of *S*. Dublin was observed.

Hygiene level*	Herd susceptibility level**	Median number of infected animals	5^{th}–95^{th} percentiles of infected animals	Median no. of ill animals	5^{th}–95^{th} percentiles ill animals	Median no. of dead animals	5^{th}–95^{th} percentiles dead animals	Median no. of abortions	5^{rn}–95^{rn} percentiles abortions
Small herd (70 cows)									
A	1	21	2-58	2	0-8	0	0-2	3	0-8
A	2	26	2-89	2	0-10	0	0-2	4	0-12
A	3	42	5-151	4	0-15	0	0-3	6	1-17
A	4	72	21-217	6	0-19	1	0-4	8	1-19
B	1	43	4-163	5	0-20	1	0-5	5	1-15
B	2	84	24-256	8	1-26	1	0-7	8	2-18
B	3	152	27-375	14	1-36	2	0-9	12	3-21
B	4	204	28-524	18	1-49	3	0-12	13	3-22
C	1	144	27-427	16	1-47	3	0-13	11	2-19
C	2	197	28-524	21	2-55	5	0-16	13	3-21
C	3	257	31-746	27	3-74	7	0-22	14	5-22
C	4	340	116-825	32	11-86	9	1-27	14	8-22
D	1	228	33-644	25	4-70	7	1-23	13	5-21
D	2	293	86-781	32	9-90	9	1-30	14	6-22
D	3	376	124-860	38	15-101	12	3-34	14	8-21
D	4	439	206-895	46	17-106	15	4-38	14	8-21
Medium sized herd (200 cows)									
A	1	83	3-265	8	0-30	1	0-5	11	0-30
A	2	183	4-553	17	0-53	2	0-10	20	1-50
A	3	445	80-1293	40	4-115	6	0-20	39	9-76
A	4	782	97-1810	63	8-154	10	1-29	48	13-84
B	1	714	85-1829	81	6-221	20	1-62	39	10-64
B	2	1496	341-2144	167	34-265	45	5-83	43	26-62
B	3	2181	627-2397	231	61-293	65	14-97	44	32-59
B	4	2367	779-2533	254	82-313	75	23-108	43	31-59
C	1	2125	667-2305	296	85-374	110	26-157	42	30-55
C	2	2308	974-2454	331	113-399	130	37-176	41	31-52
C	3	2464	1286-2588	352	160-419	145	62-190	40	31-52
C	4	2543	1558-2660	367	214-429	153	88-193	40	30-50
D	1	2340	1115-2475	369	144-434	161	59-202	41	31-52
D	2	2441	1419-2572	391	204-449	176	86-214	40	30-50
D	3	2539	2169-2649	402	307-459	184	131-221	40	30-49
D	4	2584	2417-2706	411	348-463	189	148-226	39	30-49

(Table 4 continued)

Large herd (400 cows)									
A	1	220	2-980	21	0-112	3	0-22	29	0-83
A	2	742	3-2733	69	0-287	10	0-58	68	0-158
A	3	3037	70-3769	261	5-401	47	1-93	118	10-175
A	4	3996	1032-4309	366	81-458	76	10-109	115	70-156
B	1	3817	166-4045	511	15-599	156	3-209	80	25-105
B	2	4255	3847-4441	588	457-676	201	138-259	81	64-99
B	3	4673	4449-4843	637	539-722	228	169-289	83	69-98
B	4	4883	4715-5059	678	588-767	259	199-317	82	68-99
C	1	4327	4116-4515	833	731-911	398	326-452	82	67-99
C	2	4559	4382-4734	872	783-940	425	360-476	83	69-99
C	3	4789	4629-4948	883	807-952	434	377-482	85	71-102
C	4	4928	4780-5086	900	817-961	444	383-489	84	69-100
D	1	4585	4431-4746	906	831-969	457	399-501	85	71-102
D	2	4741	4604-4887	916	848-975	466	423-508	86	71-103
D	3	4916	4783-5057	924	863-987	472	429-514	86	71-102
D	4	5025	4897-5157	933	875-989	478	436-518	85	71-100

* The hygiene level affects the number of contacts (k) in the model at shown in Table 2: A=Good, B= Average, C=Poor, D= Very poor.

** The herd susceptibility level affects the susceptibility of the animals in the age groups as shown in Table 1

The number of abortions displayed an interesting pattern. At good hygiene and low herd susceptibility levels the number of abortions was low. However, in medium and large herds at good hygiene level and high susceptibility levels the number of abortions was in fact slightly higher than at poorer hygiene levels, and the number of abortions appeared to hit a plateau in all herd sizes even though the number of infected animals kept increasing when the hygiene level became poorer and the herd susceptibility increased.

Sensitivity analysis of probabilities for becoming carriers and super shedders

The results of the sensitivity analyses are shown in Table 5. In the eight scenarios without any persistently infected animals (No C and no Su), the duration of infection became unrealistically short (e.g. 5^{th}–95^{th} percentiles of duration: 3-179 weeks across all herd sizes and all hygiene and herd susceptibility levels). In the scenarios with carriers but without super shedders, the duration was shorter than but much closer to the originally modelled scenarios shown in Table 3.

Discussion

We have developed a stochastic, mechanistic and dynamic model that simulates spread of *S*. Dublin infection in dairy herds under Danish production conditions upon introduction of one infectious heifer to the herd. We found that hygiene and herd susceptibility clearly affected both the probability of spread of infection upon introduction, duration of infection, probability of extinction and epidemic size. Herd size was important for the duration, probability of extinction and epidemic size, but not for the probability of spread of infection in the herd upon exposure. These results have implications for

control of *S*. Dublin in dairy herds. In Denmark, a surveillance programme has been in place for *S*. Dublin since 2002, and infected cattle herds are encouraged to control the infection and prevent spread of infection from their herds. The prevalence of test positive dairy herds has been decreasing from 25% to 10% since 2002. However, since mid 2010 the prevalence has been hovering around 10%, and mainly large herds appear to have problems getting rid of the infection. Our model results suggest that herds really need to optimise hygiene and reduce the number of potentially infectious contacts between cattle and from the environment, and such procedures become increasingly important the larger the herd is.

Furthermore, we found indications that *S*. Dublin super shedders might be rare or non-existent, but long-term persistently infected carriers that shed lower levels of bacteria than acutely infected cattle most likely contribute to the *S*. Dublin infection dynamics that we commonly see in infected dairy herds. Future simulations will illustrate the effect of testing and managing or culling persistently shedding carriers as part of the control strategy in dairy herds.

Model assumptions

The model is based on assumptions regarding both the population being exposed to *S*. Dublin, the contact structures in the barn and infection and effect parameters. In order to reduce the number of effects to consider in this paper, we only introduced the infection in one way to the herds, namely by purchase of one acutely infected, but asymptomatic, breeding heifer. In real life, a farmer may purchase other age-groups of animals or more than one infectious animal once or repeatedly over a period of time. The infections would most likely have lasted longer, had we simulated purchase of more infectious cattle, but might have died out faster if introduced into smaller groups than the breeding heifers, which are often housed in large groups in Danish dairy cattle. Previous studies have shown that purchase of cattle and other potential contacts to infected cattle from other herds is a significant risk factor that elevates the risk of introduction of *Salmonella* to dairy herds markedly (van Schaik et al., 2002; Nielsen et al., 2007b). Thus, our results are most relevant for herds with good external biosecurity.

A total of 24 susceptibility parameters and 20 parameters determining probabilities of clinical disease, mortality and abortion were used in the Dublin-Simherd model (Table 1). The uncertainty and variability regarding the infection parameters such as duration of infectiousness were reflected by use of distributions rather than fixed probabilities. The assumptions regarding the effects of the disease (morbidity, mortality and abortions), were obtained from literature as described in section 2.3. The uncertainty associated with these parameters was not reflected in the model. Therefore, the numbers of ill and dead animals, and the number of abortions reported in Tables 4 and 5 could be biased. The results were discussed with experts with experience from *S*. Dublin outbreaks and control and were considered plausible. However, future research is required to explore the epidemiological effects concerning animal health and infection dynamics, and production effects of *S*. Dublin in infected herds in more detail.

Comparison to results of other simulation models

Although the methodology used in other studies that modelled dynamics of *Salmonella* infections in cattle differs from the Dublin-Simherd model, we have compared our results to these studies and to data from field studies and empirical knowledge about *S.* Dublin infections in dairy herds (Xiao et al., 2005; Chapagain et al., 2007; Xiao et al., 2007; Lanzas et al., 2008). Xiao et al. (2005) developed mathematical models of *Salmonella* infection in bovine dairy herds to study host, pathogen and environmental factors for within-herd infection dynamics, persistence and factors that might describe some of the differences between *Salmonella*-serotypes in dairy herds. They used SIR-models to estimate and compare basic reproduction numbers, R_0 , in four groups of cattle (unweaned calves, weaned calves, dry and lactating cows). Transmission of infection in their model could occur directly, via the environment and by pseudo-vertical transmission (periparturient) between dam and neonatal calf. The model accounted for pathogen-induced mortality and host immunity to the infection. Estimation was done using non-linear differential equations with uncertain parameter estimates from previous studies. Simulations with different settings of those parameters illustrated their effect on R_0 which affected the behaviour of infection over time. Thus, many elements in that model were similar to our model. In the model by Xiao et al. (2005) indirect transmission and lack of removal of pathogens, e.g. by neglecting to remove faeces from the barn environment, were found to result in higher numbers of infected animals and larger epidemics than in cleaner barn environment. This supports our findings that the level of hygiene was associated with both the duration of transmission upon introduction, and with the number of infectious, diseased and dead animals. The effects of multiple control strategies including strict management to improve hygiene and reduce susceptibility, vaccination, test-and-cull and test-and-manage strategies will be explored further in subsequent studies using the developed Dublin-Simherd model.

Lanzas et al. (2008) developed a mathematical, deterministic SIR model that was able to distinguish between the effects of clinically and sub-clinically infected cattle. Furthermore, they tested the effect of long-term shedders (equivalent to carriers in the Dublin-Simherd model) and super shedders on the infection dynamics. Contrary to our results, they found that carriers did not have much impact on the transmission of *Salmonella* whereas the presence of super shedders did. This discrepancy might be due to differences in assumed infectivity of the carrier and super shedder states. Furthermore, in our study all acutely infected animals were considered as equally infectious as long as they were in the acute state, so the effect of being clinically ill on transmission was mainly associated with higher probabilities of becoming super shedders and carriers. This might be true for *S*. Dublin more than for other serotypes of *Salmonella*, because *S*. Dublin has a tendency to be shed in lower concentrations in faeces than e.g. *S*. Typhimurium. Lanzas et al. (2008) did not differentiate between different serotypes of *Salmonella*. We observed an interesting pattern with increasing number of abortions when susceptibility went up at good hygiene level, whereas for poorer hygiene levels the number of abortions appeared to find a plateau for each herd size (Table 4). We investigated the count data from all iterations, and discovered that this phenomenon can be explained by a dampening effect on new infections among cows after the first 2-3 years in herds with large epidemic sizes which is also evident in the graph for cows in Fig. 5. This dampening effect is caused by a high proportion of animals that become infected several times as calves and growing heifers and therefore are resistant when they

become adult. Note that the total number of new infections is not dampened to the same extent under poor hygiene and high susceptibility circumstances, because there are still a lot of acute infections on-going among the susceptible young stock (Table 4).

Table 5 Investigation of the effect on duration, number of infected and ill animals in the Dublin-Simherd model with changed probability of becoming carriers (C) and removal of super-shedders (Su) from the model. The estimates were derived from 1000 iterations of 10-year periods following introduction of one heifer with asymptomatic acute *S.* Dublin-infection one month prior to parturition and should be compared to the predicted outcomes in Table 3 and Table 4.

Settings for C and Su		Hygiene level	Herd susceptibility	Proportion of infected herds	Mean herd infection duration in weeks**	5^{th}–95^{th} percentiles of duration**	Median number of infected animals**	5^{th}–95^{th} percentiles of infected**	Median no. of ill animals**	5^{th}–95^{th} percentiles ill animals**
Small herd (70 cows)										
No C and	no Su	A	1	0.60	11	3-22	16	2-32	1	0-4
		B	2	0.85	17	9-31	31	21-95	4	0-13
		C	3	0.94	19	10-28	112	28-122	13	3-21
		D	4	0.98	17	12-22	121	105-125	16	8-22
C* but	no Su	A	1	0.60	44	3-113	17	2-36	1	0-5
		B	2	0.84	95	11-214	52	22-180	6	1-20
		C	3	0.96	177	51-399	218	29-533	22	3-53
		D	4	0.98	245	79-520	356	124-843	35	14-88
Medium sized herd (200 cows)										
No C	and no Su	A	1	0.64	20	3-38	60	2-105	5	0-13
		B	2	0.85	34	14-63	276	90-353	32	8-50
		C	3	0.95	24	18-33	365	350-380	48	36-61
		D	4	0.99	19	15-26	376	370-391	50	39-62
C* but	no Su	A	1	0.64	80	4-173	62	2-147	6	0-15
		B	2	0.86	292	87-520	797	98-1791	80	10-185
		C	3	0.94	462	191-520	2366	826-2520	285	98-344
		D	4	0.99	501	327-520	2584	1565-2700	360	200-410
Large sized herd (400 cows)										
No C	and no Su	A	1	0.63	26	3-50	105	2-186	10	0-21
		B	2	0.83	55	27-127	571	279-1234	75	37-156
		C	3	0.95	41	20-119	704	681-1509	98	80-238
		D	4	0.98	44	17-179	726	708-2375	103	85-348
C* but	no Su	A	1	0.62	94	3-218	100	1-269	9	0-29
		B	2	0.87	470	191-520	3802	1044-4097	431	114-505
		C	3	0.95	518	520-520	4784	4581-4966	784	683-868
		D	4	0.99	519	520-520	5021	4871-5186	885	808-949

*1.5% of asymptomatic acutely infected become carriers, 45% of acutely infected, clinically ill become carriers

** Estimated for those of the 1000 herds (iterations) where spread of *S.* Dublin was observed.

Abortions only occur in breeding heifers and cows. Therefore the dampening effect is relevant for the *S.* Dublin induced abortions and the number of new infections in adult cattle (Fig. 5). We have not been able to find other studies that included *S*. Dublin induced abortions in theoretical, epidemiological transmission models. However, Carrique-Mas et al. (2010) found that abortion was the predominant clinical sign reported in 26.7% of 1348 adult cattle diagnosed with *S*. Dublin infection in an observational study of *Salmonella* in cattle from UK between 2003 and 2008, whereas other clinical signs in adults were rare. Visser et al. (1997) found that losses associated with the extra abortions in relation to *S*. Dublin in dairy herds lead to the largest economic losses together with mortality and veterinary costs.

The Dublin-Simherd model differs from previously published models by its object-based, mechanistic nature that is based on direct contact structures between individual cattle in the simulated barn sections and by its incorporation of an indirect feedback mechanism in the herd dynamics. This allows the model to keep track of each animal in each barn section and thereby model the infection and population dynamics directly for each weekly time step. This intuitive model construction is an advantage when attempting to explain the model to farmers or cattle advisors.

Comparison to results of field studies

Veling (2004) found that 27/49 dairy herds that experienced an outbreak of *S.* Dublin in a field study in the Netherlands had evidence of being persistently infected 14 months after the outbreak, and other studies have suggested that bovine dairy herds can be persistently infected with *S*. Dublin for several years (Wray et al., 1989; House et al., 1993; Nielsen, 2003). The proportion of herds that becomes persistently infected might be higher in Denmark than in the Netherlands, because the Danish dairy herds are on average markedly larger than the Dutch dairy herds, and larger herd size has been associated with lower probability of recovery (Nielsen et al., 2007b). This supports the simulation results from the Dublin-Simherd model (Table 3) in which it was found that the average duration in herds with *S.* Dublin infection ranged from less than one year to approximately 6 years in small herds and up to 10 years in large herds strongly dependent on hygiene and herd susceptibility levels.

We have not been able to find studies that suggest how high the risk of herd infection might be upon introduction of one infectious animal. In Nielsen et al. (2007b) test negative dairy herds had purchased cattle from one or more test positive dairy herds in 1,014 out of 40,812 (2.5%) quarters of the year which increased the unadjusted probability that these would change from test negative to test positive for *Salmonella* in the following year-quarter from 2.5% to 16.4%, indicating that spread of *Salmonella* had occurred in the herd. However, it is not possible to directly relate this to the risk of infection to the purchase of one infectious animal, because not all animals that are purchased from test positive herds are infectious. Accordingly, we focused on those herds where spread of infection did occur. The next step is to include economic effects and test-strategies in the model. When this is done economic assessment of different control strategies will become feasible, and the model will be used as a decision support tool for farmers, advisors and the cattle industry.

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PAPER X

Gross margin losses due to *Salmonella* Dublin infection in Danish dairy cattle herds estimated by simulation modelling

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Abstract

Salmonella Dublin affects production and animal health in cattle herds. The objective of this study was to quantify the gross margin (GM) losses following introduction and spread of *S.* Dublin within dairy herds.

The GM losses were estimated using an age-structured stochastic, mechanistic and dynamic simulation model. The model incorporated six age groups (neonatal, pre-weaned calves, weaned calves, growing heifers, breeding heifers and cows) and five infection stages (susceptible, acutely infected, carrier, super shedder and resistant). The effects of introducing one *S.* Dublin infectious heifer were estimated through 1000 simulation iterations for 12 scenarios. These 12 scenarios were combinations of three herd sizes (85, 200 and 400 cows) and four management levels (very good, good, poor and very poor). Input parameters for effects of *S.* Dublin on production and animal health were based on literature and calibrations to mimic real life observations. Mean annual GMs per cow stall were compared between herds experiencing within-herd spread of *S*. Dublin and non-infected reference herds over a 10-year period.

The estimated GM losses were largest in the first year after infection, and increased with poorer management and herd size, e.g. average annual GM losses were estimated to 49 euros per stall for the first year after infection, and to 8 euros per stall annually averaged over the 10 years after herd infection for a 200 cow stall herd with very good management. In contrast, a 200 cow stall herd with very poor management lost on average 326 euros per stall during the first year, and 188 euros per stall annually averaged over the 10-year period following introduction of infection. The GM losses arose from both direct losses such as reduced milk yield, dead animals, treatment costs and abortions as well as indirect losses such as reduced income from sold heifers and calves, and lower milk yield of replacement animals. Through sensitivity analyses it was found that the assumptions about milk yield losses for cows in the resistant or carrier stages had the greatest influence on the estimated GM losses. This was more influential in the poorer management scenarios due to increased number of infected cows.

The results can be used to inform dairy farmers of the benefits of preventing introduction and controlling spread of *S.* Dublin. Furthermore, they can be used in cost-benefit analyses of control actions for *S.* Dublin both at herd and sector level.

Introduction

Salmonella Dublin is a host adapted pathogen of cattle (Wray and Sojka, 1977; Uzzau et al., 2000). It can cause illness mainly characterised by diarrhoea, pneumonia and death in calves and adult cattle (Vandegraaff and Malmo, 1977; Greene and Dempsey, 1986) as well as abortion and decreased milk yield in cows (Morton, 1996; Carrique-Mas et al., 2010; Nielsen et al., 2012b). The infected animals can become persistently infected carriers that shed the bacteria intermittently in their faeces for prolonged periods (Spier et al., 1990; Wallis, 2006). *S.* Dublin has been reported to

survive for long periods in the environment, e.g. in wet and dried faeces (Findlay, 1972; Plym-Forshell and Ekesbo, 1996) and to persist in cattle herds for several years (Clegg et al., 1986; Boqvist and Vågsholm, 2005).

The effects on production and other economic effects of *S.* Dublin in dairy herds have not been well quantified. The economic effects can be estimated as losses, which are missed benefits (e.g. discarded milk or reduced milk yield due to disease) or costs which are the sum of losses and control expenditures (McInerney et al., 1992; Rushton et al., 1999). Expenditures are extra resources used as a consequence of the disease (e.g. veterinary fees and disease control measures). Bazeley (2006) estimated the costs of a *S.* Dublin outbreak in a dairy herd consisting of approximately 100 cows. The clinical effects such as abortions and decreased milk yield in cows, and diarrhoea and death among calves lasted for approximately two months. During this period, the costs due to the outbreak were estimated to be approximately £7870 of which almost £3600 were due to decreased milk yield. Visser et al. (1997) estimated the average losses due to *S.* Dublin infection per farm in 40 dairy herds to be around 5000 Dutch guilders corresponding to approximately 2250 euro for the period of infection. They included extra veterinary and labour costs in the losses.

The milk yield in diseased cows has been reported to decrease markedly or even stop entirely in some cases (John, 1946; Vandegraaff and Malmo, 1977), but there are few reports quantifying the milk yield losses in infected cows without clinical signs. Bazeley (2006) investigated an outbreak in a 100 cow herd with average yearly milk yield of 7000 L per cow. Abortions were the main clinical sign of *S.* Dublin in that herd, and the estimated total loss in milk yield was 19430 L over a period of approximately two months. Nielsen et al. (2012b) investigated changes in energy corrected milk yield (ECM) in 3- month intervals at cow level for parity 1, 2 and 3 and older cows (3+) following sudden high increases in *S.* Dublin antibodies directed against O-antigens in the bulk-tank milk indicative of new *S.* Dublin infection in the herd. They found that the mean daily milk yield was deceased by 1.4 Kg ECM per cow for the first parity cows during the period 7-15 months after the estimated herd infection date, while it was reduced by 3.0 Kg ECM per cow per day in the same period for parity 3+ cows. Parity 2 cows mainly had reduced yield 13-15 months after the estimated herd infection date.

In 2007, a Danish *S.* Dublin control programme was initiated. As part of the programme all dairy herds are tested for *S.* Dublin antibodies in the bulk-tank milk every three months and classified into three categories (Anon., 2009). The aim of the programme is to eradicate *S.* Dublin from the Danish cattle population by the end of 2014. It requires compliance from farmers in infected herds to reach this goal. As part of the programme, advice on control of *S.* Dublin has been communicated to the farmers. Studies have shown that it is possible to control *S.* Dublin with management changes (Jensen et al., 2004; Nielsen and Nielsen, 2012; Nielsen et al., 2012c), and that it is unlikely that culling of active carriers alone will lead to effective control (Veling, 2004). Control efforts have to be implemented over months to years to effectively control and possibly eradicate *S.* Dublin from the herd (Jensen et al., 2004; Nielsen and Nielsen, 2012). This means that control of this infection can be costly, and it is therefore necessary to get an overview of the total losses that the infection causes in the herd in order for farmers to decide on control options. Furthermore, it is in the interest of the cattle industry to know the economic losses associated with both outbreaks and consecutive endemic infections, and potential benefits associated with

control and eradication of this infection in the dairy sector in order to prioritise and plan future the disease control strategies.

It is difficult to estimate the economic and production effects of *S.* Dublin under different production conditions based on observational data, mainly because it is almost impossible to obtain good information about the infection stages of individual cattle over time. Secondly, the duration of an infection and the following production effects takes several years. This makes a before- and after herd infection comparison of economic results blurred by various other changes in management and the production system over this period. Instead simulation studies can be used to estimate the economic herd effects. Previous simulation studies of *Salmonella* have focused on transmission parameters within the herd as well as introduction and persistence of the infection (Xiao et al., 2006; Nielsen et al., 2007; Lanzas et al., 2008; Chapagain et al., 2008). Bergevoet et al. (2009) investigated cost and cost-effectiveness compared to the reduction in herd level *Salmonella* prevalence of different national control strategies for Dutch cattle herds at national level. However, there were no estimations of losses associated with the disease at herd level in that study.

The objective of this study was to estimate the gross margin (GM) losses upon introduction and spread of *S.* Dublin in Danish dairy herds up to 10 years after introduction of the infection. The results are important for farmers and farmers' organisations when evaluating the potential benefits of preventing and controlling *S.* Dublin infection in dairy herds.

Materials and methods

Structure of the previously developed Dublin-Simherd model

The "Dublin-Simherd" model used in this study is a further development of the Simherd model, which is a stochastic, mechanistic and dynamic simulation model (Østergaard et al., 2000). The Simherd model has been developed to simulate the real situation in Danish dairy herds and incorporates the complex feedback mechanism between feeding, reproduction and culling. It is used to simulate the production and state changes of animals, including young stock, in dairy herds in discrete weekly time steps. Individual discrete events (e.g. death, disease, heat detection, conception etc.) are triggered stochastically using random numbers from relevant distributions. A large set of variables describing general management are specified to represent typical management of a dual-purpose (milk and meat) dairy cattle herd of large breed (i.e. Danish Holstein or Danish Red). These are described in Østergaard et al. (2003). Simherd is used commercially for herd health consultancy and more information about the model is available at: www.simherd.com (accessed 26th July 2012).

The basic Dublin-Simherd model which reflects the within-herd epidemiology of *S*. Dublin infection was described in detail by Nielsen et al. (2012a). Briefly, in the model the population dynamics are mimicked by simulation of individual objects (animals) stored in computer memory in one of six age groups in each time step: Neonatal (0 to 7 days), pre-weaned calves (1-7 weeks old), weaned calves (8-22 weeks old) growing heifers (23 to 59 weeks old), breeding heifers (60 weeks old to first calving) and cows (from first calving until culling or death). Superimposed on this herd structure, animals are virtually allocated to one of five infection stages: Susceptible, acute infection, super shedder, carrier and resistant. The probability that susceptible animals become acutely infected depends on contact structures, age-dependent susceptibility of the individual and

number of infectious animals in the barn section and in the whole herd. The duration of each infection stage are determined by relevant distributions derived from literature and data from field studies (Nielsen et al., 2012a). The duration of the resistant stage is doubled each time the animal is infected. The number of infectious contacts is determined by four hygiene levels, and herd susceptibility is characterised by one of four levels, where each level is specified by six susceptibility parameters, one for each of the six age groups in the herd (Table 1). Animals in the younger age groups in the model are assumed to be more susceptible to *S.* Dublin than older age groups. We refer to Nielsen et al. (2012a) for the details about these parameters. The model keeps track of the infection stage of every animal in each weekly time step. In Nielsen et al. (2012a) the number of deaths and abortions as well as infected and clinically ill animals during a 10-year period after the introduction of the infection were reported for each of the six age groups in 48 scenarios representing all the combinations of three herd sizes, four hygiene and four susceptibility levels.

Table 1 Overview of the *S*. Dublin management level classifications very good, good, poor and very poor specified according to hygiene and susceptibility levels for each of the six age groups used in the Dublin-Simherd model described in detail in Nielsen et al. (2012a).

	Susceptibility at the first exposure to *S*. Dublin / number of effective contacts per week per animal for each age group					
Age groups	Neonatal calves	Pre-weaned calves	Weaned calves	Growing heifers	Breeding heifers	Adult cows
Management level[a]						
Very good	0.9 / 0	0.8 / 0	0.7 / 1	0.5 / 1	0.4 / 2	0.25 / 2
Good	0.95 / 1	0.9 / 2	0.8 / 2	0.6 / 2	0.5 / 3	0.3 / 3
Poor	1 / 2	0.95 / 2	0.9 / 3	0.7 / 3	0.6 / 4	0.4 / 4
Very poor	1 / 3	1 / 3	1 / 4	0.8 / 4	0.7 / 5	0.5 / 5

[a] Number of effective contacts per week per animal originating from environmental *S*. Dublin contamination of the herd: Very good: 0.02, Good: 0.05, Poor: 0.1 and Very poor: 0.15

Development of the model to include the effects of S. Dublin on milk production

The effects of *S.* Dublin on milk yield of individual cows in the Dublin-Simherd model were calibrated to obtain the same herd level pattern for milk yield losses in parities 1, 2, and higher parity cows found by Nielsen et al. (2012b) who modelled milk yield for 18 months after estimated time of herd infection. There were some indications that the estimated *S*. Dublin introduction dates were set up to 6 months too late in that study, so we calibrated milk yield losses to match the pattern and size observed for a 2 year period after a new assumed infection date in the three parity groups in the 28 case herds investigated in Nielsen et al. (2012b). Dublin-Simherd milk yield losses were calibrated for 85 cow herds, the average herd size in that study, and herds with hygiene and susceptibility levels corresponding to poor management (see below). In data from Nielsen et al. (2012b), it was found that the first parity cows lost on average 1200 kg ECM during the 2 years after herd infection. Second parity cows lost on average 1900 kg ECM and third or higher parity cows lost on average 2100 kg ECM during the period. When modelling the milk yield losses, the acutely infected cows were divided into acutely infected with clinical signs and acutely infected without clinical signs (Table 2). The super shedders were modelled to have the same milk

yield losses as acutely infected without clinical signs, and carriers were modelled to have the same milk yield losses as resistant cows. The susceptible cows were assumed not affected by *S.* Dublin being present in the herd.

Table 2 The percentage of lost energy corrected milk yield (ECM) for cows in infected herds compared to that of cows in non-infected herds used to model the production effects of *S*. Dublin in the Dublin-Simherd. The table includes losses used for default scenario (best estimate obtained through calibration of model settings to fit observations from 28 case herds) and for sensitivity analysis (minimum and maximum estimates).

		Parity 1	Parity 2	Parity 3+[1]
Acutely infected (clinically ill)				
	Minimum	30%	30%	30%
	Best estimate	70%	71%	73%
	Maximum	90%	90%	90%
Acutely infected (not clinically ill), or super shedder				
	Minimum	10%	10%	10%
	Best estimate	30%	31%	33%
	Maximum	50%	50%	50%
Resistant or carrier				
	Minimum	0%	0%	0%
	Best estimate	7%	8%	10%
	Maximum	20%	20%	20%

[1]parity 3 and higher

Simulations of the economic effects of S. Dublin

The following effects of *S.* Dublin were included in the model: the risk of animals becoming infected and the risk of becoming clinically ill if infected (specified for each of the six age groups), the mortality of the clinically ill animals (specified for each of the six age groups), the milk yield losses (specified separately for the acutely infected clinically ill, the acutely infected not clinically ill/super shedders and resistant/carriers), the abortions and the treatment costs. The risk of infection, the risk of becoming clinically ill and the mortality of the clinically ill animals were all assumed highest for the youngest calves and lowest for the adult cows as specified in Nielsen et al. (2012a). The mortality was dependent on whether the animal was treated or not. It was assumed that the farmer would recognise 75% of the clinically ill animals and that these would all be treated resulting in a lower probability of dying (Table 3). The mortality was affected differently by treatment between age groups. For example, the mortality for clinically ill neonatal calves was assumed to be 85% for non-treated calves and 75% for treated calves, while it was 10% for clinically ill non-treated cows and 5% for treated cows. The prices used in the calculations of gross margin were based on 2011 levels and were obtained from the Knowledge Centre for Agriculture (Table 4). The economic effects of milk yield depression due to salmonella infection implied lost income from the milk production, less feed consumption and an increased culling risk for low-yielding cows. The economic effects of increased abortions and increased calf mortality were a lack of calves and heifers in the following years, less feed costs for young-stock in that period, but

also lost income from sale of pregnant heifers or increased costs for purchasing pregnant heifers. The economic effects of increased cow mortality were costs for incineration, loss of the dead cows production which was replaced by the milk production of a first-parity-cow (often lower-yielding) when she was ready to enter the herd. A replacement heifer was not always ready at once, and thus the production of one animal could be missing for a period.

Table 3 The minimum and maximum parameter estimates concerning probability of disease, mortality and abortions used in the sensitivity analyses of model assumptions in the Dublin-Simherd model used to model the production effects of *S*. Dublin.

Parameter	Neonatal calves	Pre-weaned calves	Weaned calves	Growing heifers	Breeding heifers	Adult cows
Probability of clinical disease in acutely infected						
Minimum	0.10	0.10	0.05	0.05	0.05	0.05
Maximum	0.80	0.80	0.50	0.30	0.30	0.30
Probability of dying if clinically ill from *S*. Dublin and not treated						
Minimum	0.50	0.40	0.10	0.05	0.05	0.02
Maximum	0.95	0.85	0.60	0.30	0.30	0.30
Probability of dying if clinically ill from *S*. Dublin and treated						
Minimum	0.50	0.30	0.10	0.02	0.02	0.01
Maximum	0.95	0.80	0.50	0.30	0.30	0.30
Probability of abortion if acutely infected						
Minimum	NA	NA	NA	NA	0.02	0.02
Maximum					0.15	0.15

The effects of *S.* Dublin introduction into dairy herds were modelled in the following way: One infectious heifer without clinical signs was introduced into the herd four weeks before calving. Due to stochasticity and depending on specified management, the infection could then spread to one or more animals (including its own calf in the week it was born), or not spread at all. We simulated three herd sizes: 85 (mean herd size in the 28 case herds in Nielsen et al. (2012b)), 200 (medium sized Danish dairy herd) and 400 cows (large sized Danish dairy herd). From the original 16 combinations of herd hygiene and susceptibility levels simulated in Nielsen et al. (2012a), four were used for simulations in this study. These were classified as very good, good, poor and very poor management level, corresponding to the best, two intermediate and the worst hygiene and susceptibility levels from Nielsen et al. (2012a). This resulted in 12 scenarios (one for each of the herd sizes and each of the four management levels) and 1000 iterations were performed for each scenario. Variables specifying management in general (e.g. heat detection efficiency, feeding level) were kept equal and constant across all simulations. The management levels in this study therefore only concerned the herds' and animals' risk of becoming infected with *S.* Dublin and not the general management as such to be able to discern the effects of *S*. Dublin infection from other effects of changed management. Only iterations in which the infection spread from the introduced heifer were used in further analyses of GM losses, and estimates were summarised per year. No specific control efforts directed against *S.* Dublin were included in the simulations.

Table 4 Prices and costs used to economically quantify the simulated results of the scenarios

Variable	Value
Milk price (euro/100 kg ECM[a])	34
Feed price (euro/FU[b])	
Mixed ration, lactating cows	0.19
Roughage, calves and heifers	0.13
Concentrates, calves	0.2
Milk replacer (euro/kg)	2.7
Bull calf (euro/14-days-old-calf)	100
Slaughter value cows (euro per kg live body weight)	0.94
Pregnant heifer (euro)	1260
Open heifer (euro)	590
Dead cow (euro costs for destruction)	70
Dead heifer (euro costs for destruction)	20
Dead calf (euro costs for destruction)	6.6
Artificial insemination (euro /AI)	16
Treatment of one *S*. Dublin clinically diseased calf 0-49 days old (euro);	36
Treatment of one *S*. Dublin clinically diseased calf 50-154 days old; (euro)	30
Treatment of one *S*. Dublin clinically diseased calf/heifer/cow older than 154 days (euro)	70

[a] ECM = energy corrected milk

[b] FU = feeding unit (1 FU = 7.89 MJ of net energy for lactation)

The GM was in this study defined as annual income minus variable costs. To estimate the GM losses attributed to *S.* Dublin, discounted GM in euros and ECM were compared to 1000 replications with the same management settings and herd sizes, but where no infectious heifer was introduced to non-infected herds. A discount rate of 5% was used. The GM per cow stall for the non-infected herds was calibrated to be similar to the GM per cow stall in Danish large-breed herds in December 2011 (Knowledge Centre for Agriculture, Cattle, 2012). The GM losses were summarised per cow stall rather than per cow, because the herd size varied the first years after herd infection due to increased slaughter of low yielding *S.* Dublin-infected cows.

Sensitivity analyses

Sensitivity analyses were performed in order to evaluate which input parameters were most influential on the results of the simulations. In the sensitivity analyses, we used the herd size 200 and included all four management levels. For each of the four management levels, the 10 different settings mentioned below were used, resulting in 40 scenarios that were compared to the non-infected herd. Firstly, three scenarios were simulated to assess the effects on the GM of changed assumptions regarding the milk yield losses. These included 1) assuming no milk yield loss in the resistant and carrier cows, 2) assuming no milk yield loss in the acutely infected cows without clinical signs and super shedders, and 3) assuming no milk yield loss in the acutely infected cows with clinical signs. Next, four scenarios were modelled in which the disease effects associated with *S*. Dublin were excluded: 4) assuming no *S*. Dublin-associated abortions, 5) assuming no *S*. Dublin-associated calf mortality, 6) assuming no *S*. Dublin-associated mortality in adult cows and 7) assuming no clinical disease effects of *S*. Dublin including associated treatment costs and mortality. The best available estimates for the milk yield losses (Table 2) were used in scenarios 4-7. Lastly, the GM was estimated by using 8) assumed minimum realistic estimates from literature of all effects, 9) assumed maximum realistic estimates and 10) assumed best estimates, except

that milk yield effects were set to the assumed minimum realistic estimates. The estimates used for the input parameters in the model used in sensitivity analysis scenarios 8 to 10 can be seen in Tables 2 and 3.

Results

Simulation results

For the reference herds, the simulated annual mean GM per cow stall averaged over 10 years with no infectious heifer introduced were 1088 (5th to 95th percentiles: 848 to 1354), 1131 (890 to 1373) and 1108 (894 to 1343) euros per stall for 85, 200 and 400 cow stall herd, respectively (Fig. 1). Due to the way the model was specified there was no difference in the annual mean GM between the *S*. Dublin related management levels for the respective herd sizes in the reference herds. The mean annual milk yield averaged over 10 years was 9482 (9233-9727), 9647 (9483-9809) and 9589 (9472-9707) Kg ECM per cow per year for 85, 200 and 400 cow herds, respectively.

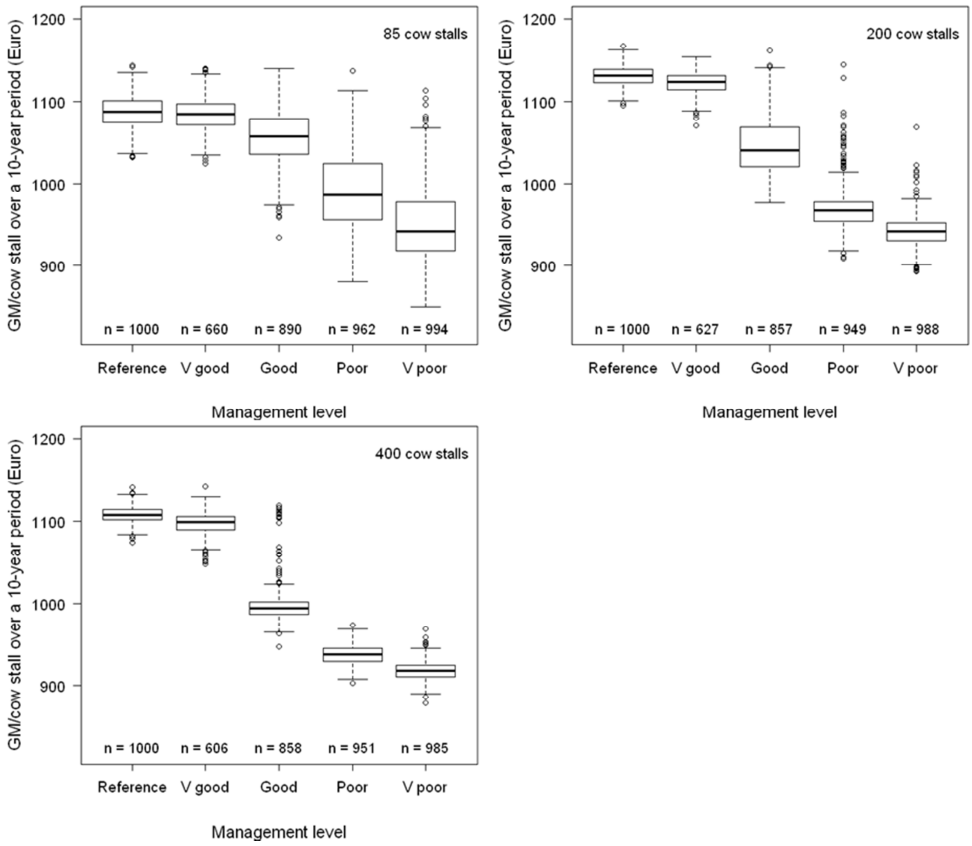

Figure 1 The model predicted annual mean gross margin (GM) per cow stall in euro averaged over the 10 years after *S.* Dublin herd infection for three herd sizes (i.e. 85, 200 and 400 cows) and four management levels (i.e. Very good, Good, Poor and Very poor). Estimates were derived from 1000 iterations, and n denotes the number of model iterations in which spread of *S*. Dublin occurred in the infected herds (except for the reference herds, where it denotes the 1000 iterations run without infection). These were the iterations used to calculate the losses in GM.

The estimated number of infections in animals and duration of herd infection in herds with spread of infection were reported by Nielsen et al. (2012a). For very good management the *S.* Dublin infection spread within the herds in 600 to 627 iterations for the three herd sizes. The number of iterations with spread of infection increased to 995 iterations for very poor management (Fig. 1). The simulated annual mean number of infections in herds with 200 cow stalls can be seen in Fig. 2. The number of annual infections can be greater than the herd size, because animals can become infected more than once per year, if infection is still present in the herd after they return to the susceptible state.

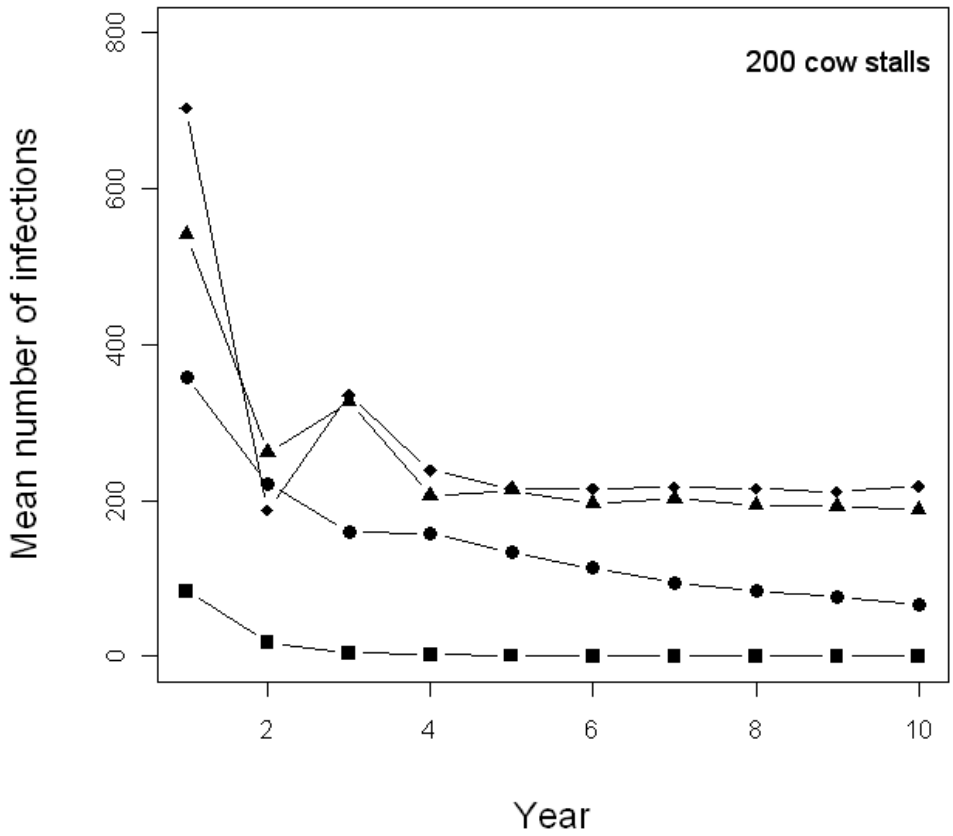

Figure 2 The model predicted mean annual number of *S.* Dublin infections in simulated dairy herds with 200 cow stalls (multiple infections occurred in some animals). Estimates were derived from 1000 iterations of the 10 years following introduction of one infectious heifer and only iterations in which spread of *S*. Dublin occurred were used. (■) corresponds to very good, (●) good, (▲) poor, and (♦) very poor management.

The management level influenced how long the infection persisted in the herd with the mean number of infected animals reaching 0 in year four after introduction of *S*. Dublin in the very good management level. For poor and very poor management levels, the mean annual number of infections stabilised at around 260, i.e. the infection rarely went extinct from poorly managed herds. The estimated losses in ECM were correlated to the number of infections, and the poorer management levels were estimated to have the greatest and most prolonged losses (Fig. 3).

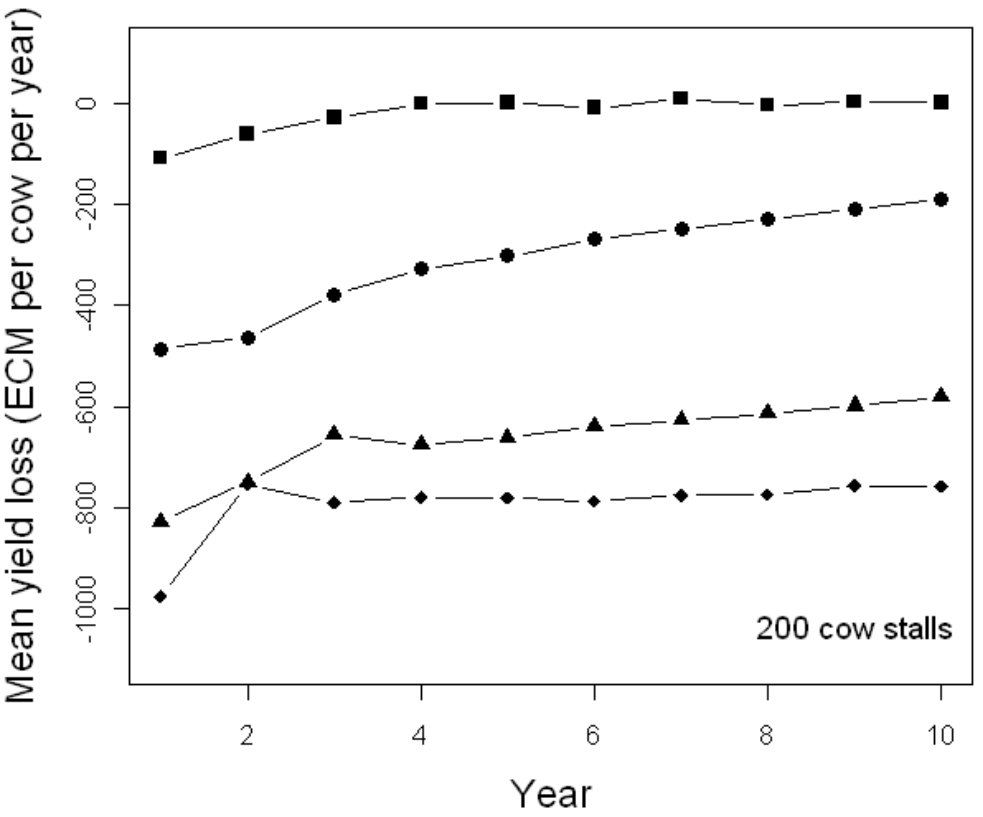

Figure 3 The model predicted difference in mean annual energy corrected milk yield (ECM) per cow between *S.* Dublin infected and non-infected reference dairy herds with 200 cow stalls (mean yield losses per cow). Estimates were derived from 1000 iterations of the simulated 10 year period after introduction of infected heifer into the herd. (■) corresponds to very good, (●) good, (▲) poor, and (♦) very poor management.

The estimated annual mean GM per cow stall was 1123 (892 to 1349), 1047 (841 to 1251), 968 (764 to 1183) and 942 (738 to 1190) euros for very good, good, poor and very poor management, respectively, in the 200 cow stall herd averaged over the 10 years after introduction of infection (Fig. 1). Similarly, the differences in annual mean GM per cow stall between infected and reference herds averaged over the 10 years were estimated. With very good management, the GMs were similar in infected and reference herds. For very poor management the GM per stall in herds with 85 cow stalls was -137 (-316 to -37) euros in infected herds compared to reference herds, -188 (-331 to -99) euros in 200 cow stall herds and -190 (-324 to -98) euros in infected vs. reference herds with 400 cow stalls.

Figure 4 illustrates the GM losses per year over the simulated 10-year period. Generally, the GM losses were greatest in the first year after herd infection. However, the GM losses increased from year three to four after the herd infection date for good, poor and very poor management in the 200 and 400 cow stall herds. This corresponds to an increase in the number of animal infections in year three compared to year two (Fig. 2).

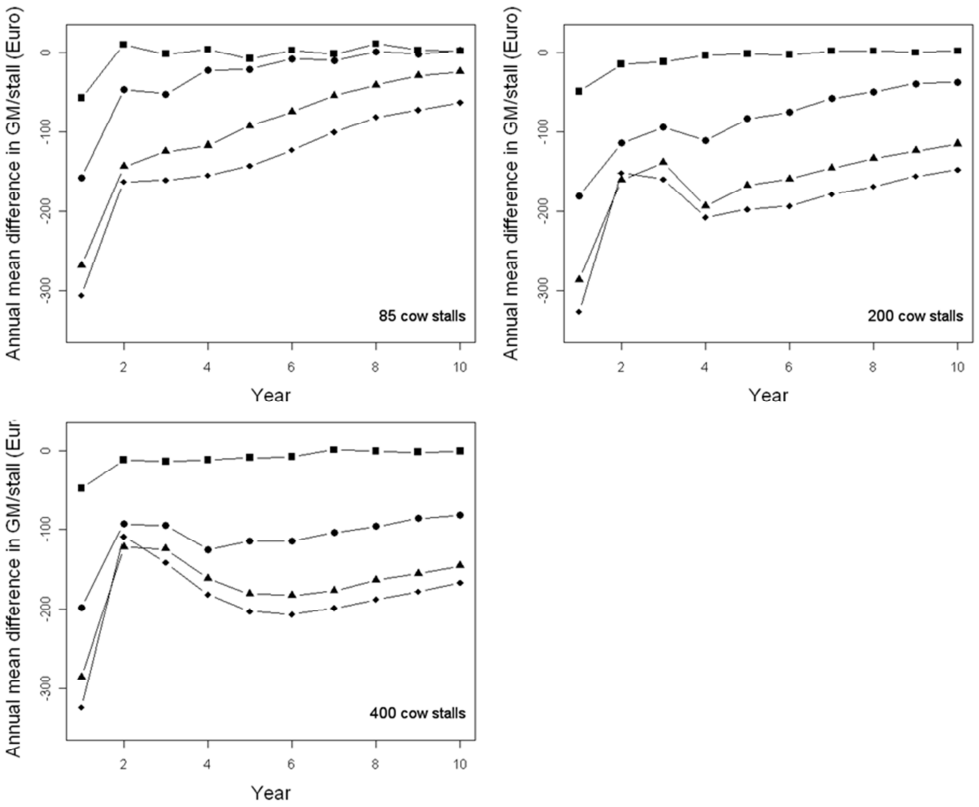

Figure 4 The model predicted difference in annual mean gross margin (GM) per stall in euro between infected herds and non-infected herds during the 10 years after *S.* Dublin herd infection for three herd sizes (i.e. 85, 200 and 400 cows) and four management levels. Estimates were derived from the number of iterations provided in Fig. 1. (■) corresponds to very good, (●) good, (▲) poor, and (♦) very poor management.

Table 5 gives an overview of foregone returns and decreased costs for calves, heifers and cows in the first year as well as averaged over 10 years after herd infection for the four management scenarios. It can be seen that income from milk has the highest absolute values followed by feed for cows and young stock.

Sensitivity analysis results

Table 6 provides the sensitivity analysis estimates of the GM losses per stall in the first year after *S.* Dublin herd infection and the average annual GM losses per stall over the 10 years after the herd infection date in a 200 cow stall herd. It can be seen that the mean GM losses per stall in the first year for the best estimate scenario were 41 euros for very good management. Likewise, the mean

GM losses per stall were on average 7 euros per year in the 10 years after herd infection for this scenario. The annual mean GM losses per cow stall averaged over 10 years were less than the losses in the first year after the herd infection date independent on management levels and the magnitude of the effects simulated in the sensitivity analyses. Increasing all *S.* Dublin effects to assumed maximum realistic estimates resulted in higher GM losses per stall the poorer the management level. Similarly, reducing all milk yield effects of *S.* Dublin by 50% reduced the GM losses per stall more for very poor management scenarios than for very good management scenarios. This followed the same pattern when simulating no milk yield losses in the resistant or carrier cows which reduced the GM losses more, the poorer the management.

Table 5 Overview of the difference between annual income and expense variables used in the Dublin-Simherd model for estimation of economic losses associated with *S*. Dublin during the first year, and annual averages over a 10-year period after the herd became infected with *S*. Dublin in a 200 cow stall herd and under four management scenarios.

	Difference from the mean value of the reference herds over the 1st year (euro per cow stall)				Difference from the mean value of the reference herds averaged over 10 years (euro per cow stall per year)			
Economic variable	Management				Management			
	Very good	Good	Poor	Very poor	Very good	Good	Poor	Very poor
Income from milk sold to the dairy	-57	-233	-379	-434	-9	-101	-208	-250
Income from slaughtered cows	-10	2	17	19	0	1	-1	-4
Income from sold heifers	3	4	5	4	-1	-24	-53	-64
Income from sold bull calves	-2	-6	-9	-11	0	-1	-2	-3
Expenses for cow feed	-17	-68	-108	-122	-3	-27	-55	-66
Expenses for young stock feed	1	-3	-10	-13	0	-13	-41	-56
Expenses for purchase of replacement heifers	1	23	45	50	0	3	5	7
Expenses for insemination of cows	-1	-1	-2	-2	0	0	-1	-1
Expenses for treatment of cows (not salmonella-related disease)	-1	-4	-6	-7	0	-1	-2	-3
Expenses for treatment of salmonellosis cases	2	9	13	15	0	2	5	5
Other expenses cows[a]	-2	-8	-13	-14	0	-2	-3	-5
Other expenses young stock[a]	1	2	1	2	0	-1	-4	-5
Displacement balance[b]	0	-2	-3	-4	0	-2	-5	-7

[a] Ear tags, administration fees, disposal fees etc.

[b] Changes in herd value from beginning to end of year

The mean GMs per stall and the 5th and 95th percentiles for the sensitivity scenarios, the best estimate (results shown in Fig. 1 and 4) and the reference herd in absolute values are shown in Fig. 5 averaged for the 10 years after the herd infection date. Simulating no yield losses in the resistant and carrier cows, no yield effects at all or reduction of all effects resulted in relatively small averaged GM losses per stall over 10 years compared to the reference herds for good, poor and

very poor management (Fig. 5). All simulated sensitivity scenarios resulted in relatively small GM losses per stall for very good management over the 10 years after herd infection. Only when all effects were increased did it result in much greater losses than any other scenario for very good management level.

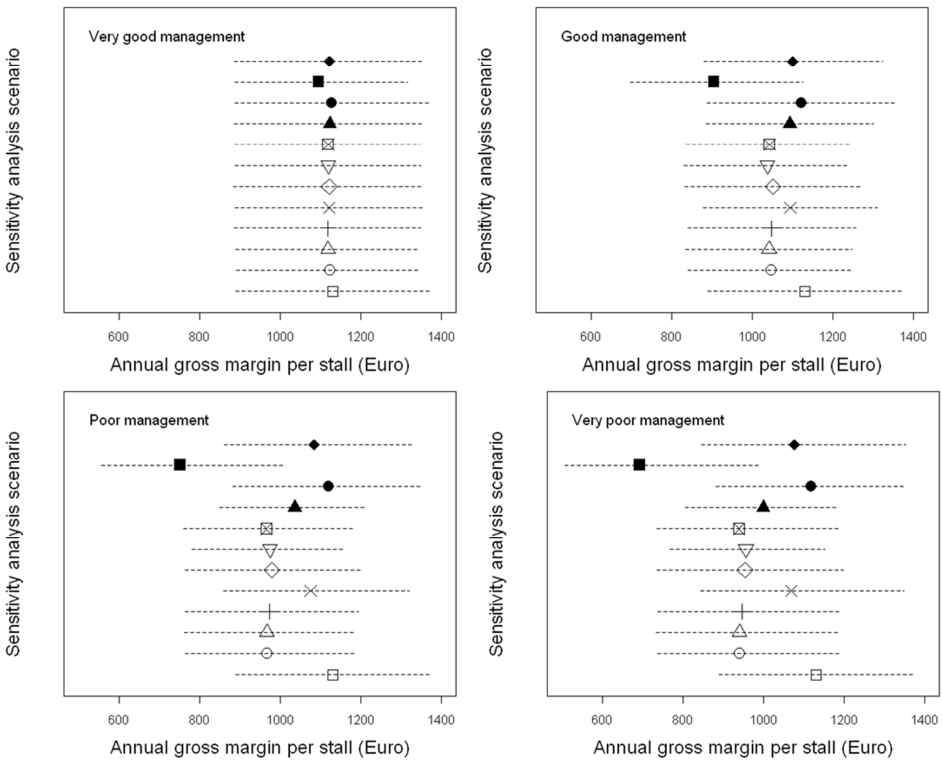

Figure 5 The model predicted differences in mean annual gross margin (GM) per stall between *S.* Dublin infected and non-infected dairy herds under the assumptions used in the sensitivity scenarios specified in Table 2, and the best estimate scenario. Estimates were averaged over 10 years in a 200 cow stall herd in euros. Estimates were derived from those model iterations in which spread of S. Dublin occurred out of 1000 (estimates for reference herds were based on 1000 iterations). (□) is GM for non-infected reference herd, (○) for best estimate, (Δ) no milk loss acute infected and diseased, (+) no milk loss acute infected not diseased/supershedders, (x) no milk loss resistant/carriers, (◊) no abortions, (v) no dead calves/heifers, (☒) no dead cows, (▲) no clinical effects of infection, (●) all effects reduced, (■) all effects increased, (♦) all yield effects reduced by 50%. The dashed lines show the 5th and the 95th percentiles from the simulations.

Discussion

To our knowledge this is the first study quantifying both the direct and the indirect economic effects of *S.* Dublin in dairy herds. Hence, this study provides valuable information in relation to disease control programmes for *S*. Dublin. The fact that the estimates were specified for different herd sizes and management level makes it feasible to use these to obtain sector level estimates of

the impact of this infection. Individual farmers can use the results to get an idea about how much he/she is losing per year on having *S*. Dublin in the herd.

Table 6 The model predicted differences in mean annual gross margin (GM) per stall between *S.* Dublin infected and non-infected dairy herds under the assumptions used in the sensitivity scenarios specified in Table 2, and for the best estimate scenario. Estimates are given in euros for the first year and averaged over 10 years in a 200 cow stall. Estimates were derived from those model iterations in which spread of *S*. Dublin occurred out of 1000 and compared to 1000 simulations of non-infected reference herds.

	Change in GM per stall (euros) from non-infected reference herd							
Management level	Very good		Good		Poor		Very poor	
Assumptions	1st year	10 years	1st year	10 years	1st year	10 years	1st year	10 years
Best estimate	-41	-7	-151	-78	-245	-151	-284	-174
No milk loss acute infected and diseased	-48	-12	-167	-89	-257	-164	-296	-190
No milk loss acute infected not diseased/ supershedders	-47	-12	-163	-84	-256	-157	-282	-183
No milk loss resistant/carriers	-38	-9	-112	-37	-168	-56	-194	-62
No abortions	-25	-10	-120	-79	-192	-151	-221	-177
No dead calves/heifers	-50	-12	-184	-93	-290	-156	-337	-175
No dead cows	-51	-12	-175	-88	-275	-165	-315	-192
No clinical symptoms of infection[a]	-39	-8	-139	-37	-239	-95	-268	-131
All *S.* Dublin effects reduced[b]	-6	-4	-20	-8	-29	-12	-34	-13
All *S.* Dublin effects increased[b]	-101	-36	-462	-227	-674	-379	-745	-438
All milk yield effects reduced by 50%	-36	-8	-84	-31	-122	-46	-139	-54

[a] No clinical symptoms and no deaths, hence no treatment costs either. Milk yield losses still present

[b] Parameter estimates displayed in Table 2

Management levels in this study were based on severalfactors such as herd hygiene, which is affected by e.g. thecleaning practices to reduce the amount of manure buildup in different barn sections of the herd and sectioning ofage groups, as well as herd susceptibility which is affected by colostrum handling and feeding practices and animal density among other things (Nielsen et al., 2012a). In order to improve the management level and reduce the effects of *S*. Dublin herd

infection, it is possible that simple and cheap measures such as improving calving and colostrum management and sectioning of pre-weaned calves would suffice in some herds, whereas in other herds it might need to be combined with improved hygiene practices among all age groups, changed purchase patterns and culling of sus-pected carriers, which may be more costly (Nielsen et al., 2012c, Nielsen and Dohoo, 2011). However, further simulations are needed to evaluate the cost-benefit of different control strategies for different types of farms.

The GM losses associated with *S.* Dublin infection were quantified by simulation modelling. The results in this study were based on the milk price in Denmark in December 2011. The average 10-year GMs estimated for the reference herds were evaluated to correspond to the current level of GMs observed in Danish dairy herds when taking into account the discounting over 10 years. It is, however, possible that potential future changes in the milk price could change the economic losses estimated here. Results in this study estimated greater losses than previous studies. The Dublin-Simherd model was calibrated to field data estimating that milk yield was affected for at least 18 months after herd infection, and simulations estimated that the milk yield was decreased even longer than this for many of the scenarios. Hence, milk yield was affected much longer than the two months that Bazeley (2006) used for estimating losses. Visser et al. (1997) found lower economic effects of *S.* Dublin infection but they included herds after isolating *S.* Dublin from samples, which means that they did not necessarily include newly infected herds like we simulated in this study. This would result in expected lower losses than what was found in our study, where the outbreak phase was included.

Results

The annual GM losses per cow stall increased with increasing herd size and with decreasing quality of management specifically relevant for *S.* Dublin. This indicates that it is even more important to control *S.* Dublin in large herds, and that more resources can be spent on the control efforts here than in smaller herds. The increased effects in the large herds were partly due to the infection persisting in the herds and partly due to a greater number and proportion of the animals in the herds becoming infected. For very poor management, there was no difference between losses in 200 and 400 cow stall herds. This is likely due to the fact that the very poor management level sustained the herd infection at similar high prevalence in these two herd sizes. The fact that herd sizes and management levels affecting spread of *S*. Dublin were included in this study makes it possible to use the results to inform farmers of the potential benefit of eradicating *S*. Dublin from their herds. The within-herd prevalence and incidence of specific herds can be estimated from repeated diagnostic testing in the herd (Nielsen, 2013), and this can be used to indicate which management level is relevant for the herd in question.

In order to achieve the milk yield reduction following *S.* Dublin herd infection that was observed in data used by Nielsen et al. (2012b), it was necessary to model milk yield losses into the resistant stage of the infection cycle in the individual animals. Nielsen et al. (2012b) reported that milk yield at herd level appeared to be returning to pre-infection levels approximately 15 months after estimated time of herd infection. In contrast to this, Bazeley (2006) reported milk yield losses for a period of approximately two months. Other types of *Salmonella* have been reported to affect milk yield for shorter periods of time, e.g. *S.* Anatum for four months (Glickman et al., 1981) and six months for *S.* Typhimurium (Kahrs et al., 1972). The effects in our study appeared to be lasting longer even for the 85 cow herd. This might be explained by the fact that we assumed that no

control efforts were implemented in the infected herds and the management level was kept constant during all 10 years. This was done to separate the effects of the infection from the effects of the control efforts. In real life, some control efforts were most likely implemented in herds experiencing an outbreak of *S*. Dublin, both in the herds investigated in the study by Nielsen et al. (2012b) and other studies. Control efforts could shorten the period with active *Salmonella* infection in the herds by management changes, and potentially lead to lower yield losses in infected animals through intensified treatment or isolation of sick animals. Finally, culling of sick or suspected carrier animals may have been used in some herds in relation to *Salmonella* outbreaks described in the literature, which would decrease the period where *Salmonella* affected milk yield (Bergevoet et al., 2009; Nielsen and Dohoo, 2011; Nielsen and Nielsen, 2012). The Dublin-Simherd model also includes culling of animals, but only related to production and reproductive performance, not related to the *S*. Dublin infection stages.

The GM losses estimated in the sensitivity analyses indicated that no single effect of *S.* Dublin (e.g. abortion or milk yield losses in resistant or carrier cows) determined the GM losses averaged over the 10 years when the management was very good. However, for the poorer management scenarios, the assumptions regarding milk yield losses in resistant or carrier cows influenced results markedly. The infection died out within a relatively short time period in the very good management scenarios and this reduced the overall number of resistant cows in the herd over the 10 years. This group of animals was large in the poorer management scenarios, where the herd infection persisted longer, resulting in greater GM losses per cow stall. However, the sensitivity analyses also showed that even if we overestimated the milk yield losses in cows, there were still substantial economic losses associated with introduction and spread of *S*. Dublin in dairy herds. In addition to the milk yield losses, feed prices were influential on the results of the simulation model. These were not included in the sensitivity analyses since the price was not an effect of *S.* Dublin herd infection.

Method

The only cost of *S.* Dublin herd infection included in this study was the treatment of clinically ill animals. Other costs such as extra labour and disease control procedures (except treatment costs) were not included. Hence, the effects of *S.* Dublin on the GM per cow stall were reported as losses, even though technically they could be defined as costs of infection (McInerney et al., 1992).

The number of iterations used from infected herds was lower than for reference herds, because we only used the iterations in which spread of *S*. Dublin occurred. This, however, does not affect the results to any noteworthy extend, because the distributions of GM losses would be very similar for e.g. 600 iterations and 1000 iterations. It would not make sense to include the iterations in which spread of infection did not occur upon introduction of an infectious heifer, when estimating the effect of *S*. Dublin infection in the herds.

The GM and the milk yield for all non-infected reference herds were identical independent of management level. This was due to the definition of the management levels in this study, which were based exclusively on the risk of infection with *S.* Dublin. However, this is unlikely to reflect the real situation, where management that can be considered poor management with regard to *S*. Dublin might also lead to lower milk yield and lower GM due to other uncontrolled diseases, such as mastitis and paratuberculosis (Gröhn et al., 2004; Lombard et al., 2005). Hence, potential bias in

the results comparing reference and infected herds warrants care in the interpretation. It is difficult to say, whether the GM losses have been overestimated under poor management conditions, so further investigations including observational studies in herds with an without different diseases and management levels are recommended.

For very good management, it appeared that the GM per stall decreased when omitting the single effects in the sensitivity analysis. This was due to feedback mechanisms in the model. For example, if no or few infected cows died, these cows would stay in the herd and contribute with less milk than a healthy replacement animal and lower the GM in the actual scenario. This illustrates the advantage of using a simulation model that mimics natural feedback mechanisms in dairy herds. The next step is to use the model to simulate actual control scenarios and decide on cost-effective ways of controlling *S.* Dublin in herds of different sizes.

The effect of introduction of *S.* Dublin on milk yield was based on Nielsen et al. (2012b). In that study, the milk yield was modelled for 18 months after estimated herd infection. There were indications in data that milk yield decreased earlier than estimated by Nielsen et al. (2012b), which indicate that the infection date might have been estimated to be later than what actually was the case. Hence, we used milk yield losses over 2 years to calibrate milk yield effects in the present study. However, it is possible that this has over- or underestimated the milk yield effects of infection and thereby the estimated losses in GM. The sensitivity analyses showed that the assumptions regarding milk yield losses were important for the estimates of GM losses associated with *S*. Dublin, and further studies are needed to quantify the effect on milk yield in individual cows in different infection stages to validate the findings of this study.

Estimated milk yield losses were calibrated at poor management level settings in the model. It is not known how management in the infected herds in the study by Nielsen et al. (2012b) corresponded to management in this study. Furthermore, the management definitions in this study were created based on hygiene levels and herd susceptibility levels, which can be difficult to translate into an actual management level. However, the herds studied by Nielsen et al. (2012b) were selected due to very high and relatively sudden increase in antibody levels in bulk-tank milk indicating that they were heavily infected and therefore a poor management level was assumed reasonable.

Only one infectious heifer was introduced in the infected scenarios in this study. It is possible that farmers purchasing animals will introduce more than one infectious animal at once, or will introduce infectious animals to the herd repeatedly over time. Particularly in herds with very good management, this could lead to greater GM losses due to more infected animals. Furthermore, the animal could be introduced to other age groups than heifers just before calving. This could lead to different infection dynamics in the herd than simulated in this study, depending on the age of the animal since younger animals are more susceptible to *Salmonella* (Hall and Jones, 1979; Segall and Lindberg, 1991), and group sizes and dynamics differ.

Reduced feed costs for diseased animals were included in the model because milk production and feed consumption are directly linked in the model. However, no labour costs were included in this study and these would further decrease GM per stall. It is, e.g. likely that diseased animals would need extra attention and that this would increase labour costs. These would need to be included in control simulations, where extra labour could be required to control the infection. The treatment

costs are dependent on the farmer's ability to discover diseased animals and threshold for when he would contact the veterinarian. These were held constant throughout the different managements in this study, and could have been included in the sensitivity analyses. They were left out of the sensitivity analyses to reduce complexity in the presentation of the study.

To summarise, the simulations in this study estimated potentially large losses in the GM per cow stall following introduction and spread of *S.* Dublin in dairy herds. The GM losses were greatest in the first year after herd infection and large herds experienced greater losses than small herds. Furthermore, poorer management resulted in greater GM losses per cow stall. Milk yield losses appeared to be the effect of *S.* Dublin that had the greatest impact on GM losses, and therefore these need to be parameterised with care in the simulation model. Further studies are needed to quantify effects of *S.* Dublin infection in cattle such as milk yield losses and probability of abortions in different *S.* Dublin infection stages of dairy cows.

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PAPER XI

Modelling a national program for the control of food-borne pathogens in livestock: the case of *Salmonella* Dublin in the Danish cattle industry

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Summary

A 'virtual hierarchy' model is described for studying the spread of pathogens between herds of livestock. This novel approach to simulating disease has animals, herds, and geographic regions in a national livestock industry arranged as a hierarchy of objects in computer memory. Superimposed on all objects is an infection-recovery cycle, a control program, and surveillance based on test results and animal movement. The model was applied to predicting progress in the control of *Salmonella* Dublin in the Danish dairy cattle industry over a ten year period. More frequent testing of bulk tank milk for antibodies to *Salmonella* Dublin was less effective than improved herd biosecurity. Restricting cattle movement between regions provided a strong benefit to those regions initially with a low prevalence of infection. Enhanced control within infected herds was of intermediate benefit. A combination of strategies was highly effective although cost and feasibility of this option needs further exploration.

Introduction

Simulation modelling can provide insight into the epidemiology and control of infectious disease in animals and man and is widely adopted as a decision support tool in many disciplines. Historically, the most common approaches to simulating the transmission of infections in populations are strongly mathematical and based on differential equations and matrix algebra. These models have been applied to many viral and parasitic diseases of man and animals (Anderson and May, 1992; Scott and Smith, 1994) and are increasingly being used to elaborate the epidemiology of human enteric pathogens derived from livestock (Turner et al., 2003; Vosough et al., 2006; Xiao et al., 2005; Xiao et al., 2006).

Reliance on models that are heavily based on mathematical processes can limit the flexibility available for dealing with complexities found in some practical settings. Complexity typically occurs, for example, when interventions to control disease are superimposed over the natural cycle of infection and recovery. In particular problems can arise when models attempt to mimic large populations since the constituent members (herds or individual subjects) are very likely to be heterogenous with respect to traits that influence infection, recovery and detection within a surveillance system. Under these circumstances models that are based on a mathematical process are often not sufficiently flexible to reflect an understanding of the system under study. As well, models with a strong mathematical basis can sometimes lack intuitive appeal amongst the practitioners of disease control because the inner workings are either not transparent, not intelligible, or both. Examples of models of infectious disease that are useful because they combine statements of logic with mathematical processes are becoming more common (Allore et al., 1998; Wood et al., 2006; Wood et al., 2007).

Because there is such a broad diversity in the types of decisions facing veterinary and medical authorities, expansion of the range of techniques available for integrating modelling and disease-control is much needed. Ideally, such models should be easily demonstrated to decision makers and be sufficiently flexible to evaluate a range of control measures that might be considered by policy makers.

In this paper, we describe the virtual hierarchy approach to simulating transmission of infection in a large and heterogeneous population. We did this by developing a model for studying *Salmonella enterica* subsp. *enterica* serovar Dublin (*S.* Dublin) infection in the population of Danish dairy cattle herds (here the level of interest is the herd). *S.* Dublin is primarily associated with cattle, causes disease and production loss in many countries, and is a problematic pathogen in dairy cattle production. The organism also infects man by the food-borne and direct contact routes and has a propensity to be rapidly invasive and cause high mortality (Vugia et al., 2004).
Since 2002, Denmark has implemented a national surveillance scheme in cattle in an attempt to reduce the public health and economic impact of *S*. Dublin. The program is based on periodic assessment of herd infection status by measurement of antibody to *S*. Dublin in bulk tank milk (BTM) by ELISA at 90 day intervals. In Denmark, all herds are continuously classified according to their risk of infection. Herds officially referred to as "Level 1" are at low risk of being infected (on average less than 1% probability that the herd is infected), "Level 2" herds are at higher risk (on average more than 80% probability that the herd is infected), and "Level 3" herds are those with culture confirmed clinical salmonellosis (very few herds are Level 3, a maximum of about 15 at any given point in time, and remaining Level 3 for approximately 3-4 months). Herds move from Level 1 to Level 2 classification if a concentration of antibody indicative of infection is detected, or, for at least a 3-week period after they purchase animals from a herd that is classified as Level 2. Herds are promoted to Level 1 when antibody concentrations decline in BTM following the elimination of infection. Herds classified as Level 2 because of a purchase from a Level 2 herd can be promoted to Level 1 if the next scheduled test for antibody in BTM following the purchase is negative. This system was introduced to discourage farmers to purchase animals from high risk herds, and the effects on trading patterns were dramatic within the first half a year after the initiation of the surveillance program. The surveillance program has been described in detail and evaluated elsewhere (Nielsen et al., 2007; Warnick et al., 2006) The aim of the current work was to develop a virtual hierarchy model of *S*. Dublin infection and control in the population of Danish dairy cattle herds by adapting knowledge of the pathogen, animal population and surveillance measures. The primary purpose of the model is to predict changes in the prevalence of herds infected with *S.* Dublin over time under different control strategies.

Methods

Overview

The initial stage of modelling involves adapting and organising existing knowledge of the epidemiology of the pathogen of interest, in this case the key features of the ecology of *S*. Dublin infection in cattle, to create a conceptual model of the pathogen at herd, regional and national levels. The conceptual model is a simplified account of the real world, obtained by considering the relationships between elements of the system that have a non-trivial influence on the occurrence of *S*. Dublin in Danish cattle herds. The second stage involves transforming the conceptual model into computer code to produce a simulation program that accepts various inputs (allowing experimentation with the model) and that provides outputs consisting of time-dependent estimates of the proportion of herds infected and the proportion of herds classified as high risk or infected (Level 2 and Level 3, hereafter collectively referred to as Level 2). Finally, the third stage involves formulating a basis for the input assumptions by collecting and organising existing knowledge (established facts and expert opinion), extracting and analysing data obtained from the surveillance of *S*. Dublin in Danish dairy cattle herds (serology and microbiology findings over time), and extracting data on herd demographics and patterns of movements of animals between

herds from the Danish Cattle Database (DCD). Each of these stages of model development is described in detail below.

Model structure

The system under study can be represented as a hierarchy consisting of groups of dairy cattle managed within a common herd, followed by groups of herds located within a common geographic region with similar prevalence of *S*. Dublin and then groups of regions comprising the entire dairy cattle industry of Denmark (over 7000 herds located in seven regions). The objective of the model is to follow each of these elements of the hierarchy through time, by monitoring changes in each herd's *S*. Dublin true infection status and risk classification, and summarising these traits at the regional and national level at the completion of each time step. In this model, the duration of the time step is a single day and we estimate the national and regional outcomes each day for the duration of an entire iteration. A single iteration may comprise any number of consecutive days, although for the purposes of informing policy on control of *S*. Dublin a maximum duration of 3650 days (ten years) is adequate. A complete simulation consists of multiple iterations with the results collected at the end of each iteration and these summarised descriptively at the end of the simulation to provide a picture of the variation of possible outcomes from the model when taking into account the stochasticity of the infection process. The regions referred to in this model and their abbreviations used in figures are listed in Table 1. These regions do not have an official status under Danish statutes but have been devised by workers in animal disease control as a useful system for classifying geographic location of herds within the country (Nielsen et al., 2007).

Table 1 Regions of Denmark referred to in the results for simulation of *S*. Dublin in cattle herds and their corresponding abbreviations.

Abbreviation	Geographic region
EJ	East Jutland
ISL	The Islands
NJN	North Jutland North (Vendsyssel)
NJS	North Jutland South (Himmerland)
NWJ	North West Jutland
SJ	South Jutland
WJ	West Jutland
DK	Denmark (all regions combined)

Herd level infection and recovery

Central to the conceptual model is the infection-recovery cycle of herds exposed to *S*. Dublin. Instead of the "susceptible-immune-recovered" (SIR) technique with subjects (herds) considered *en masse*, the current approach assumes that at each time step each herd exists in one of five non-overlapping time periods defined by the state of infectiousness and level of antibody in BTM. When arranged in their temporal order of occurrence these periods describe the infection-recovery cycle for herds (Figure 1). Herds existing in the "true-negative period" are those that that

are both free of infection with *S*. Dublin and have low levels of antibody in BTM. If a herd is exposed to a source of *S*. Dublin that leads to spread of infection within that herd then in that time step it moves from the true negative period to the "dissemination period" - a phase where *S*. Dublin is being actively disseminated throughout the herd but as yet there are insufficient animals shedding the organism in faeces for the herd itself to be regarded as infectious and antibody levels in BTM have not increased. At the conclusion of the dissemination period the herd enters the "antibody lag period" when a proportion of the herd (defined by within herd prevalence) is actively shedding the pathogen and clinical signs of a new outbreak are usually evident. If any such "shedding" animals are sold to a clean herd they may cause a new outbreak of *S*. Dublin. In the "antibody lag period" there has not yet been a detectable rise in the level of antibodies in BTM (the herd is effectively 'false-negative' if BTM is tested for antibodies in this period). Once the level of antibodies in BTM rises sufficiently high, the herd enters the true positive period and it remains a source of infection for other herds if it participates in trading. Finally, at the end of the infection-recovery cycle, the herd enters the "antibody fall period" during which *S*. Dublin has been eliminated from the herd but antibody levels in BTM persist ensuring the herd remains classified as Level 2 if a test is scheduled. At the conclusion of the antibody fall period, antibody in BTM reverts to normal (low) levels and the herd once again enters a true negative period. Herds in the true-negative period stay there indefinitely until exposed to a source of infection upon which the cycle begins again.

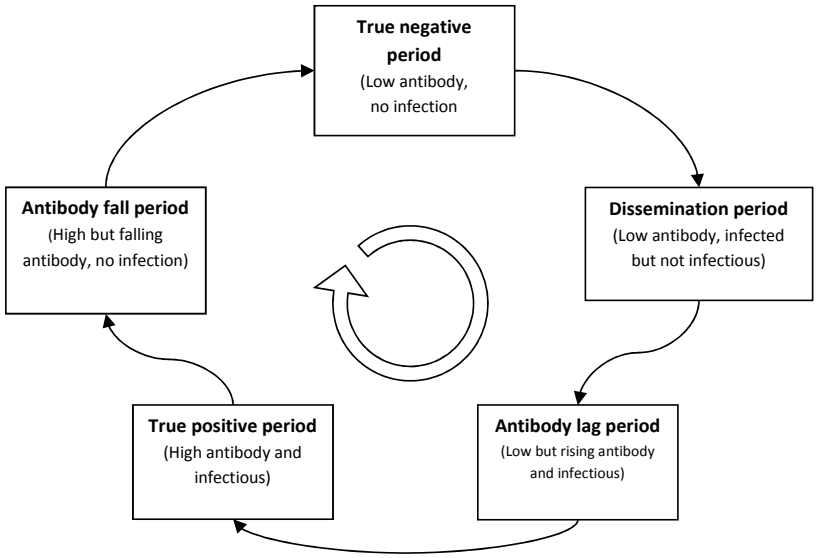

Figure 1 Diagram of the infection-recovery cycle of *S*. Dublin in Danish dairy cattle herds used to model the temporal changes in surveillance status of herds and their true infection status.

By incorporating the above infection-recovery cycle into the model the infection status, infectiousness (shedding) status and test status of each herd can be followed through time. Thus, for example, if animals are moved from a herd that is in either the antibody lag period or the true positive period there is a possibility that at least one of these animals can transmit *S*. Dublin to the purchasing herd. However, in this model such a movement would have no impact if there are no infected animals in the consignment or the receiving herd is itself already infected (in both cases

no new outbreak would result). Similarly, it is a simple matter to know the infection classification of each herd by keeping track of the time-steps during which they have high levels of BTM antibody.

Movement of infected cattle

Livestock trading is inevitably a complex issue owing to the many human, economic, regulatory and production influences that govern decisions to buy or sell. Consequently, the conceptual model adopted a simplification of the trading behaviour of Danish dairy herds by defining all herds according to the following three attributes: (*a*) number of days per year that livestock are purchased, (*b*) the number of cattle that are acquired per purchase event (assuming there is no more than one purchase event per day), and (*c*) the 'buying behaviour' of herds. Both (*a*) and (*b*) can be described as probability distributions with density estimates obtained from analysis of data from the DCD that records the date of all movements in and out of all herds at the individual animal level. The third variable (*c*), 'buying behaviour', is a surrogate measure of one aspect of biosecurity and classifies each herd as either 'closed' (no purchases of cattle), 'conservative' (purchases are only made from *S*. Dublin Level 1 herds) or 'indiscriminate' (herds that buy from either Level 1 or Level 2 herds). Each of these possible classifications is mutually exclusive allowing buying behaviour to be represented by a discrete probability distribution that is defined by an analysis of data on cattle movements within each of the seven regions (see below). At the beginning of each iteration all herds are assigned a buying behaviour by sampling from the discrete probability distribution for that region and this behaviour is retained by each herd until the end of the iteration such that in different iterations the same herd can have a different buying behaviour.

With each new time step in the model each herd is evaluated to see if it is required to purchase cattle by performing a single Bernoulli trial, with *p* (the probability of success) equal to the herd's pre-allocated probability of purchasing a consignment of cattle on any one day (see below). Because herds that are 'closed' are not permitted to buy animals they do not require purchasing to be simulated. Herds with a buying status that is 'conservative' are permitted to buy cattle from any herd that has a Level 1 status in that same time period. Herds that are 'indiscriminate' buyers can buy cattle from any herd (regardless of Level). For all purchase events the source herd is chosen at random from a list of the eligible herds and the number of animals purchased is also a random value from the corresponding input probability distribution. Finally, the number of infected animals in the purchase consignment is made equal to nil if the source herd is free of infection while for infected herds it is a random variate from the binomial probability distribution having parameters *p* (the within herd prevalence of infection) and *n* (the size of the consignment).

The model also includes an option to restrict the movement of animals between regions. With respect to *S*. Dublin no such restrictions are currently in place in Denmark although this could be introduced in the future and it is a common strategy for the control of livestock disease in many other animal health jurisdictions. Thus, the model includes an option to force herds that seek replacement animals to only obtain them from their own region instead of from any region.

Surveillance

In the Danish surveillance program for *S*. Dublin, dairy herds are assessed for evidence of infection approximately every 90 days by assaying BTM for antibodies using an ELISA. Previous studies have

documented a strong association between within herd prevalence of seropositive animals and infection in the herd and the level of BTM antibody response (Warnick et al., 2006; Nielsen and Ersbøll, 2005). High or rapidly elevating antibody is taken as indicative of infection and results in the herd being classified as Level 2. ELISA results from up to four consecutive samples are used to assess whether reclassification to Level 2 is required. Thus, herds may move from Level 1 to Level 2 if antibodies in BTM rise to a high level as evidenced by a single test, or, if antibodies slowly rise and persist such that the mean of four consecutive tests exceeds the critical value. In the simulation model, each herd has its testing scheduled at a set interval (the default being 90 days). At each time step, each herd is queried to establish if a test is scheduled for that day and if so it is simply a matter of identifying which period of infection or recovery the herd is in. If the herd is in the true-positive period or the antibody fall period then the surveillance test will be simulated as positive otherwise it will be negative. Herds with a positive test are immediately allocated a Level 2 status if they are not already Level 2. Herds with a negative test are kept at Level 1 or promoted to Level 1 if they were Level 2 before testing negative.

Start conditions

At the commencement of each iteration (t = 0) the population of herds is established by deriving the infection status for each herd given its BTM antibody status on 31 December 2005. From here herd infection status at t = 0 is simulated from estimates of positive predictive value and negative predictive value for the BTM ELISA (derivation of the estimates for predictive values is discussed below). The infection status of antibody negative herds is thus the outcome of a Bernoulli trial with p equal to the negative predictive value (here p describes the probability that an antibody negative herd is not infectious) and the infection status of antibody positive herds is the outcome of a Bernoulli trial with p equal to the positive predictive value.

Software implementation

The conceptual model was encoded into software using an object-oriented programming language allowing rapid development of the interface through 'drag and drop' addition of visual components (e.g. memo boxes, edit boxes and labels) onto a form from a component pallet (Borland® Delphi™ 7 for Windows®, Borland Software Corporation, Scotts Valley, California, USA). The use of object-oriented code is critical to the development of the model because it enables orderly management of the hierarchy of objects (country, regions and herds) and their associated code for the manipulation of the correct data during simulations. Other strongly object-oriented programming languages such as C^{++}, or $C^{\#}$ could also be used to develop a similar model. Central to the construction of this model is reliance on a non-visual object referred to in Delphi as TObjectList which has the ability to own and manipulate a list of any other objects. In this model, three specialised descendents of TObjectList were derived to represent each level of the hierarchy (TDenmark for the national level, TRegion and THerd). Only one instance of TDenmark was required and this held in its object list seven instances of TRegion (one for each region) with each TRegion object holding N_R instances of THerd (R = 1 to 7), where N_R is the number of herds in each region. Additional code was provided to each of the descendent classes of TObjectList specific for its behaviour in the model. For example, THerd has a procedure called "THerd.AntibodyFallPeriod" that defines the behaviour of any particular herd during the antibody fall period, TRegion has a procedure called "TRegion.RegionStep" for managing all the events that occur in a particular region within a single time step, and TDenmark has a procedure called "TDenmark.BuyFromL1" for simulating the purchase of a consignment of cattle on behalf of any herd in any region with the

source of cattle being any Level 1 herd in any region. In addition to the code for managing the object hierarchy, additional code was written for input of fixed and stochastic assumptions, setting of simulation options and the output of simulation results as text and plots. Specialised routines for obtaining random variates from probability distributions were adapted from those used in an earlier model (Jordan and McEwen, 1998) and are largely based on the techniques outlined by Law and Kelton (2000).

Simulation inputs

Prior to all simulations, default data on the population of dairy herds were loaded into the model. This described the BTM ELISA test result, the classification status (Level 1 or Level 2), and the region of origin of each herd (n = 7,401) at 31st March 2004. The information was acquired from the DCD and edited using SAS analysis software (version 9.1, SAS Institute Inc, Cary, NC, USA) then loaded into the simulation model as a flat data-base file in ASCII format.

Probability distributions describing positive and negative predictive value for deriving each herd's infection status at $t = 0$ from their BTM ELISA status at $t = 0$ were generated for herds belonging to each of the seven regions. In short, the process involved extracting from the DCD for the period 2001-2005 the distribution of BTM ELISA results from known infected and non-infected herds and correlations between consecutive ELISA tests for each herd. These findings act as inputs for a model that simulates both antibody measurements on herds at 90 day intervals and the surveillance classification levels that would result. Then the estimated predictive values for each region of interest at t = 0 were derived. The process is fully described in a related study (Warnick et al., 2006).

Time periods in the infection recovery cycle are central to the functioning of the model. Information on the epidemiology of *S*. Dublin infection in cattle in Denmark is available from earlier work using repeated ELISA testing (sera, individual milk sampling and BTM) and faecal culture applied to 12 herds. Referred to as the "Kongeå project", methodology and outcomes have been previously described (Warnick et al., 2006; Nielsen et al., 2007; Nielsen et al., 2004). Information used to inform decisions on probability distributions for each of the time periods in the infection-recovery cycle for herds consisted of evidence from the Kongeå project, theoretical knowledge of the ecology of *Salmonella* infection in individual cattle, and the combined experiences of the authors (each having had protracted involvement in field and research aspects of enteric pathogens in cattle).

The "dissemination period" equates to the period of time for an outbreak to commence in herds following the introduction of a source of infection so that such herds can be regarded a potential source of infection. This time period is variable owing to differences in the amount of infection initially introduced, herd structure and contact dynamics, variation in the amount of shedding in individual animals, time of onset and duration of shedding in individuals. It is possible to estimate a theoretical minimum for the duration of the dissemination period by assuming that: (i) herds have an average size of 80 cows and 150 animals in total, (ii) that at least 5% of animals must be infectious for the herd to be infectious to other herds, (iii) it takes on average two days for an animal to become infectious from the time they are exposed to the pathogen, (iv) individual animals are infectious for 12 days on average (Robertsson, 1984), and (v) each animal infects on average two other animals during its entire infectious period (Nielsen et al., 2007. This means that

after two days we could have three infectious animals, after four days we could have seven infectious animals and after six days we could have 15 infectious animals in the herd. However, this timing is highly unlikely because there is not free and unrestricted contact between all animals in a herd, the interval between first generation cases and second generation cases is not always as short as two days and contacts do not all occur immediately after individuals become infectious. Thus, while cognisant of the above theoretical limit, we set the minimum dissemination period for herds to 14 days to be consistent with experience in the field whereby herds rarely show signs of a new infection within 14 days of the introduction of carrier animals. A 'most likely' dissemination period of 30 days was adopted to be consistent with levels of contact that normally occur in Danish dairy herds and the typical appearance of signs of infection in herds after exposure to a source of contamination. However, in herds with limited contact between animals or groups of animals, or in herds with animals becoming infected on pasture the dissemination may well be longer. We therefore set the maximum possible duration of the dissemination period to 120 days.

The "antibody lag period" is the time it takes for the concentration of antibodies in BTM to rise above the cut-off value used in the surveillance program classification after dissemination of infection to a level of at least 5% infected animals in the herd. This rise in antibody is assessed from ELISA results from up to four consecutive tests. Experience from the field shows that this period can be quite short (approximately two weeks that it takes infected cows to produce high antibody levels in serum (Robertsson, 1984)), if the infection spreads from within the lactating cow section of the herd. However, this period can also be much longer (up to 120 days) if the infection spreads first within the calf barn and the calves and the lactating cows are housed separately. We set the most likely antibody lag period to 60 days.

The 'antibody fall period' is the time for the antibody level in BTM to fall to levels low enough for the herd to enter the Level 1 classification once there are no longer infectious animals present in the herd. We estimated the distribution of this period based on data acquired from eight dairy herds during a field study. The herds had blood samples collected from all young stock twice per year and milk samples collected every quarter of the year from lactating cows for a period of three and a half years while managers attempted to eradicate the infection through hygiene control and test-and-cull strategies. Herds were considered free of infectious animals when there were no longer any signs of new infections in the young stock. From this time to the Level 1 classification could be reached it took between 0-810 days with the most likely being around 180 days, however, it was difficult to estimate accurately due to the fairly long testing intervals in the intervention herds. Based on the above a beta-pert distribution with parameters 0 (minimum), 180 (most likely) and 810 (maximum) was used to represent the duration (days) of the antibody fall period.

The 'true positive period' is the time from when BTM antibody levels have reached high enough to classify the herd Level 2 until the herd clears the infection (the herd is infectious throughout this period). Estimation of this period is problematic because evidence of the demise of infection in herds is unobtainable due to the need for extensive and repeated faecal culture. Consequently, we used the BTM ELISA data from all herds to estimate the total duration of the high antibody period, which consists of both the true positive period plus the antibody fall period and then subtracted from this the estimate of the antibody fall period (above). The subtraction of one probability distribution (antibody fall period) from another (high antibody period) was performed by

simulation with only the non-negative simulation outputs retained for fitting to a suite of candidate parametric distributions using @Risk software (Palisade Corporation, NY, USA).

The 'high antibody period' is not formally part of the model but used above to derive the true positive period. Duration of test positive periods cannot be calculated directly from the surveillance program data because the data are censored due to most measurements having been made at 90 day intervals. Therefore an analysis of all the antibody measurements for all herds for the period of 2003 through 2005 was performed as follows. If a herd had more than one test within three months, one value was selected at random and then all herds were then classified as test-positive or test-negative at each testing event using the surveillance program criteria. If four sequential measurements for a herd spanned a period of more than 15 months (5 year-quarters), then the observations on that herd for that period were excluded. All such observations on consecutive quarters (n = 72144 from 7728 dairy herds) were then used to calculate the probability of changing from test positive to test negative and test negative to positive. We then assumed these transitions followed a first-order Markov process with the average duration of test positive status equal to the inverse of the positive to negative transition rate. Finally, the distribution of the duration of test positive days was obtained as an exponential distribution with the parameter (mean) equal to the average duration of test positives.
The DCD keeps track of all movements of cattle between herds, the date of such movements, the identity of the origin and destination herds and the number of animals involved. Extensive manipulation of the database using SAS software was undertaken to estimate probability distributions for the following input assumptions: number of purchase events per herd per year (as an empirical discrete distribution describing count data), number of animals obtained at each purchase event (also as an empirical discrete distribution describing count data), and the purchasing behaviour of each herd (as an empirical discrete distribution describing categorical data).

Data describing the prevalence of individual cattle infected with *S*. Dublin within infected herds (within herd prevalence) was obtained from the Kongeå project. In that work, faecal culture had been performed on multiple animals within infected herds on multiple occasions. We collated the results of 33 such samplings, expressed the data as a proportion of animals culture positive and then used this to derive an empirical probability distribution for entry into the model.

The environmental exposure probability (EEP) is a variable in the model that encompasses all exposures to infection other than those caused by contact with an infected animal. Exposure of livestock and man to enteric pathogens by the various environmental pathways is an insidious process that it is difficult to accurately describe and quantify. Although the literature does contain many qualitative data on *Salmonella* in the environment (for a summary see Murray (2000)) it does not contain quantitative estimates of the frequency of transfer of *Salmonella* between cattle herds by environmental pathways. Some studies specific for *S*. Dublin do also provide good qualitative evidence that transfer of *S*. Dublin does occur between cattle herds along environmental pathways but again quantitative data suitable for incorporation in a simulation model are lacking (Fossler et al. 2005; Taylor and Burrows, 1971; Vaessen et al., 1998). To overcome this deficiency on the probability of spread of infection between herds by environmental pathways we performed a calibration exercise using the model to establish an order of magnitude estimate of the daily probability that a cattle herd will be exposed to *S*. Dublin

from an environmental source. A value of EEP was obtained by searching for a value that provided an estimate of the percentage of Level 2 herds that was consistent with that observed at the planned commencement of simulation experiments (1 Jan 2006) and which did not cause the model to behave in a manner likely to be implausible given existing knowledge of the system.

Experimentation with the model

Following the initial simulations to establish a value for EEP the model was used to evaluate the current system of surveillance and control and then various modifications representing specific decisions made to enhance the control of *S.* Dublin in Danish dairy cattle in the future. Where full simulations are performed these involve 1000 iterations (trial and error had previously shown this number to be sufficient to describe the output distributions) and a descriptive graphical analysis performed on predictions of the percent of herds classified Level 2 at t = 3650 days and the percent of herds infected at t = 3650 days.

Scenario 1 is the base scenario and approximates the current management of *S.* Dublin in the Danish dairy cattle industry. It is used as a comparison for the intervention scenarios described immediately below. Inputs were defined as the default values described above and with EEP set to 10^{-5}. In addition, herds were allowed to acquire replacement animals from any other herd regardless of region by only taking into account their simulated purchase policy, and BTM ELISA testing was performed at the usual 90 day interval.

Scenario 2 simulates the effect of restricting movement of cattle so that they are confined to their own regions. This prevents high prevalence regions from 'exporting' infection thereby protecting low prevalence regions from external sources of *S*. Dublin infection. In practice, there are many possible options for controlling animal movement between regions (for example some regions may have restrictions placed on them but not others, some regions may import but not export etc.). In this scenario we merely wish to obtain a general appreciation of the extent of benefit from restricting movement between regions and so apply the restriction to all regions. This scenario is implemented by activating a switch option that was built into the model and software which forces herds seeking replacement animals to only acquire the from the herd's home region.

Scenario 3 evaluates aspects of herd-level biosecurity. In cattle production, the chance that a herd acquires an infectious agent from another herd can be reduced by restricting the number of animals that are traded, reducing the frequency of trading and adopting a policy of only obtaining replacement animals from herds regarded as a "low-risk". Notwithstanding the possibility that such practices can have a deleterious economic impact, the benefits accrued from applying this approach to the control of *S.* Dublin does need to be quantified. The "enhanced biosecurity" scenario therefore limits all herds to no more than 12 purchase events per year (by truncating the input distribution used in the base scenario at 12 purchase events per year) and limits the number of animals acquired at any one purchase to 12 (by truncating the base scenario inputs for this variable at 12 animals per trade). In addition, the distributions describing the purchase policy of herds within each region were altered as follows: both the proportion of herds with an "indiscriminate" purchase policy and the proportion of herds with a "conservative" purchase policy were halved with the remaining proportion assigned a purchase policy of "closed".

In Scenario 4 we evaluated the gains from testing herds more frequently by reducing the interval between BTM ELISA tests to 30 days (the current practice reflected in the base scenario is a 90 day

BTM test interval). Such a practice would be expected to improve the predictive values of the surveillance classification scheme.

Scenario 5 examines the effect of enhanced control of *S.* Dublin at the herd level. As the number of Level 2 herds in the Danish dairy industry is falling it might soon be feasible to direct more resources at herds as soon as they become Level 2 with the aim of hastening the elimination of the pathogen and thus increasing the pace of industry-wide control of *S*. Dublin. The effect of such measures would be to reduce the duration of time that individual herds spend in the true positive period – by responding quickly to reduce the spread and severity of infection within the herd. Thus, in this scenario we halved the mean of the exponential distribution used to model the true positive period in the base scenario so that this period was simulated as an exponential distribution with mean of 338 days. Presently, there are no data available to discern whether or not this extent of improvement in control of *S.* Dublin within herds is possible. However, the aim of this simulation was merely to obtain a general understanding of whether further investigation of this approach should be pursued. Finally, we created Scenario 6 by combining all the features of the previous four scenarios to provide some indication of the maximum possible reduction in prevalence that might occur with this composite approach.

Table 2 Probability distributions describing predictive values for the bulk tank milk (BTM) ELISA derived by analysis and used to generate herd infection status at the commencement of simulation (t = 0).

Region	Predictive value positive test	Predictive value negative test
EJ	Triangular(0.611, 0.713, 0.752)	Triangular(0.995, 0.997, 0.997)
ISL	Triangular(0.515, 0.589, 0.617)	Triangular(0.998, 0.999. 0.999)
NJN	Triangular(0.656, 0.771, 0.816)	Triangular(0.991, 0.994, 0.995)
NJS	Triangular(0.719, 0.836, 0.884)	Triangular(0.959, 0.973, 0.97)
NWJ	Triangular(0.666, 0.783, 0.829)	Triangular(0.989, 0.992, 0.994)
SJ	Triangular(0.706, 0.826, 0. 874)	Triangular(0.971, 0.981, 0.984)
WJ	Triangular(0.688, 0.808, 0.856)	Triangular(0.982, 0.988, 0.990)

Abbreviations for region names are defined in Table 1. Triangular distribution parameters are given as minimum, mode, maximum respectively.

Table 3 Input probability distributions describing duration in days of elements of the infection recovery cycle of *S*. Dublin in Danish dairy herds.

Component of infection recovery cycle	Probability distribution*
Dissemination period	Triangular (14, 30, 120)
Antibody lag period	Triangular(14,16,120)
True positive period	Exponential(726)
Antibody fall period	Beta-pert(0, 180, 810)

*Triangular and beta-pert distribution parameters are given as minimum, mode, maximum respectively. The parameter for the exponential distribution is the mean.

Results

Model inputs from data analysis

Predictive values for the BTM ELISA at $t = 0$ that were calculated for each region are shown in Table 2. Probability distributions derived and used to estimate the duration of each time period within the infection recovery cycle for all herds regardless of region are shown in Table 3. Empirical probability distributions used to estimate the number of purchase events per year for each herd and the number of animals acquired at each purchase event are shown in Figure 2.

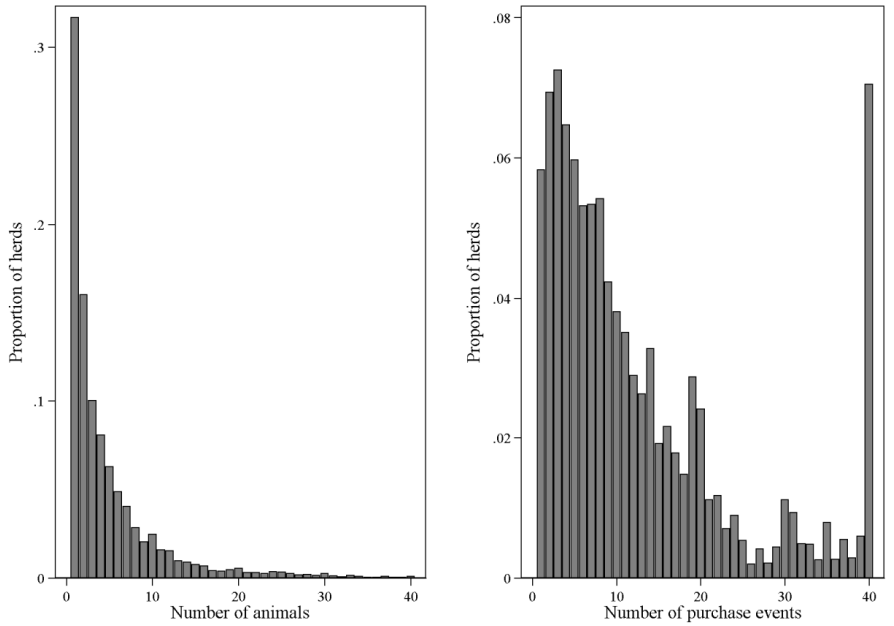

Figure 2 Discrete distributions describing a) the number of cattle acquired by herds at each purchase event, and b) the number of purchase events per herd per year.

Further exploratory analysis (using plots of various class intervals of number of purchase events per year) failed to reveal any dependency between these variables (plots not shown). The descriptive analysis of purchasing behaviour of herds in various regions is shown in Figure 3 and reveals that regions vary substantially with respect to this trait. Within-herd prevalence of infection data recovered from the Kongeå project is plotted as a probability distribution function in Figure 4.

Model outputs

Outputs from the model (prevalence of infected herds and prevalence of Level 2 herds) occur in two formats. Firstly, as time-series plots of the outputs from a single iteration of the model. This provides a picture of the behaviour of the model through time and is useful for interactive comparisons using the software (examples are Figures 5 and 6) for visualizing differences between iterations and the impact of stochastic effects. The second form of output is the results from full simulations (scenario) consisting of predictions for both outcomes (prevalence of infection and prevalence of Level 2) at a given number of days in the future and repeated for the number of iterations. The outputs are analysed using box plots for each region of Denmark and a national

summary. The box plots for all simulation scenarios are arranged in two panels (one for prevalence of infection and another for prevalence of Level 2) to illustrate the variability between and within simulation scenarios and between and within regions (Figures 7 and 8).

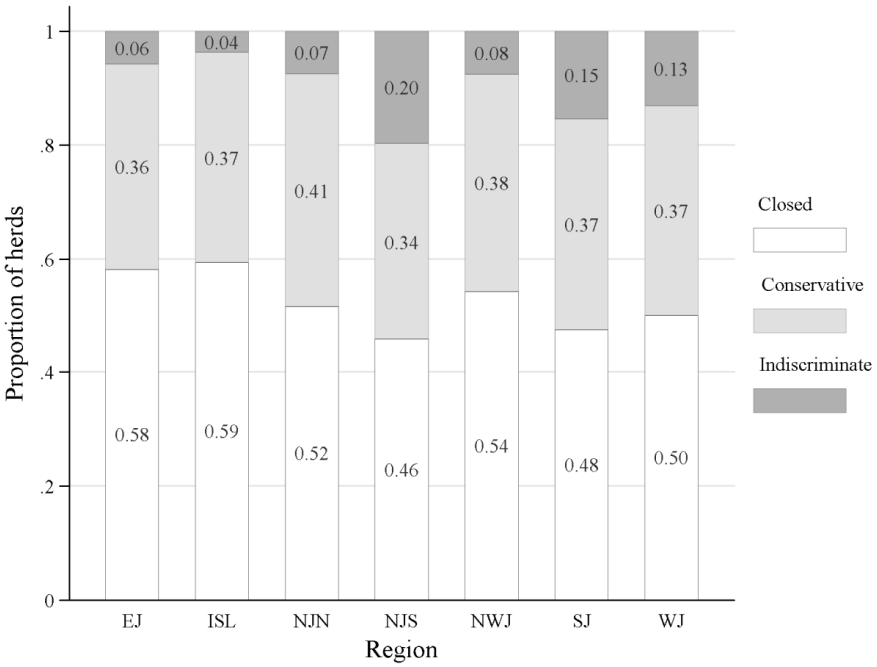

Figure 3 Composition of herds with respect to buying policy in each of seven regions of Denmark.

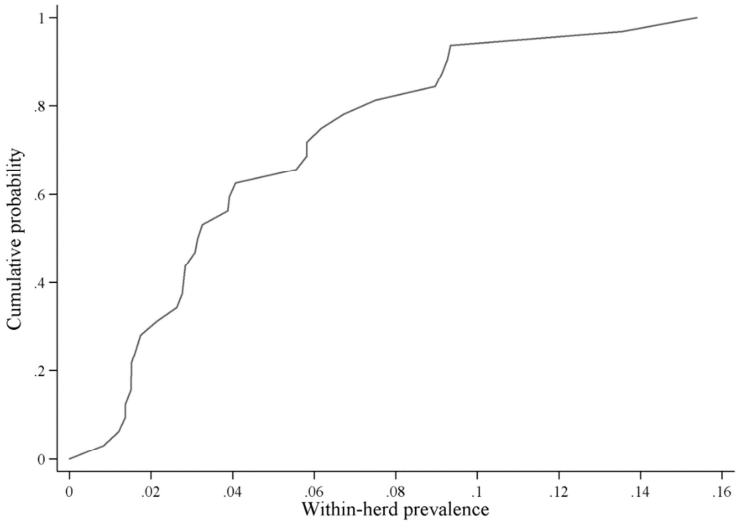

Figure 4 Input data on the within herd prevalence of infection with *S*. Dublin as an empirical distribution function, data acquired from intensive and repeated culture of faecal samples from animals in known infected herds.

Figure 5 gives outputs from single iterations of the model under the base scenario (in the form of time series plots of percent of herds classified as Level 2) with four different levels of environmental transmission (EEP input variable). This output graphically illustrates the importance of environmental transmission of *S*. Dublin in cattle, the key role of the EEP variable in the model, and why at subsequent simulations a level of EEP = 10^{-5} was used. When EEP = 10^{-3} the percent of Level 2 herds increases markedly over a three year period in a manner that is completely inconsistent with surveillance system results for recent years. When EEP = 10^{-4} the proportion of Level 2 herds is virtually static over a 10 year period. While this is possibly consistent with a static-endemic pattern of disease, it is inconsistent with surveillance data from recent years showing the percent of Level 2 herds gradually falling. In contrast, EEP has very little effect on the model at values less than 10^{-5} (Figure 5d shows the behaviour for EEP = 10^{-6} which is identical to output for whenever EEP < 10^{-6}). However, at a value of EEP = 10^{-5} the resulting time series plot is the most consistent with the downward trend in proportion of Level 2 herds that has been experienced in recent years, and for these reasons EEP = 10^{-5} was used as the level of environmental transmission in the other simulation scenarios.

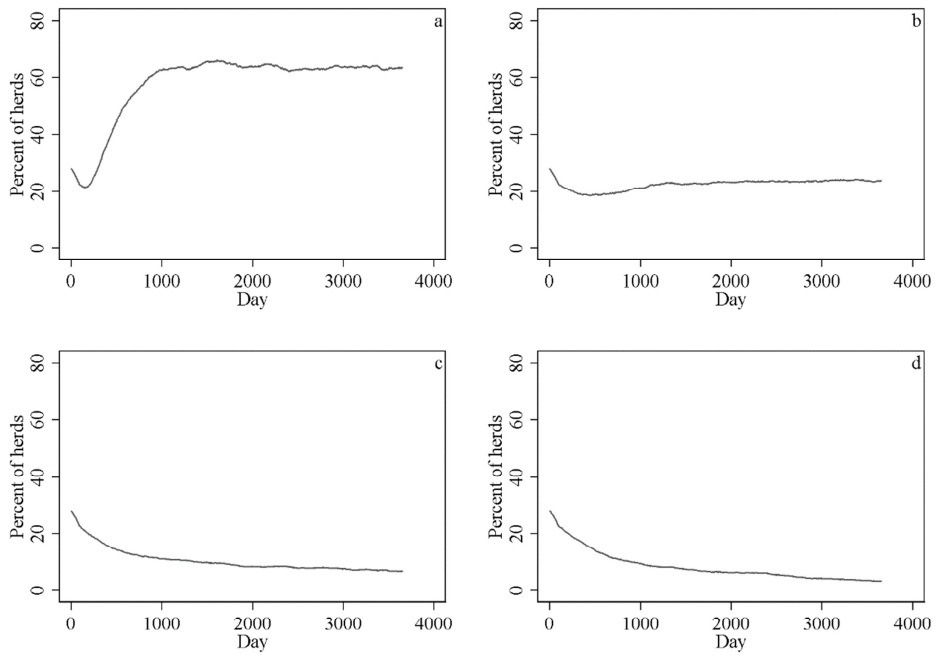

Figure 5 Output (predicted percent of herds as Level 2) from a single iteration demonstrating the effect of the input variable describing the per-herd, per-day probability of environmental exposure (EEP): a) EEP = 10^{-3}, b) EEP = 10^{-4}, c) EEP = 10^{-5}, and d) EEP = 10^{-6}. The results for EEP = 10^{-6} are representative of results for EEP < $10^{-.55}$ (additional plots not shown).

An illustration of the behaviour of the model is given by time-dependent predictions of the proportion of herds classified as Level 2 and the proportion of infected herds from a single iteration of the base scenario (Figure 6).

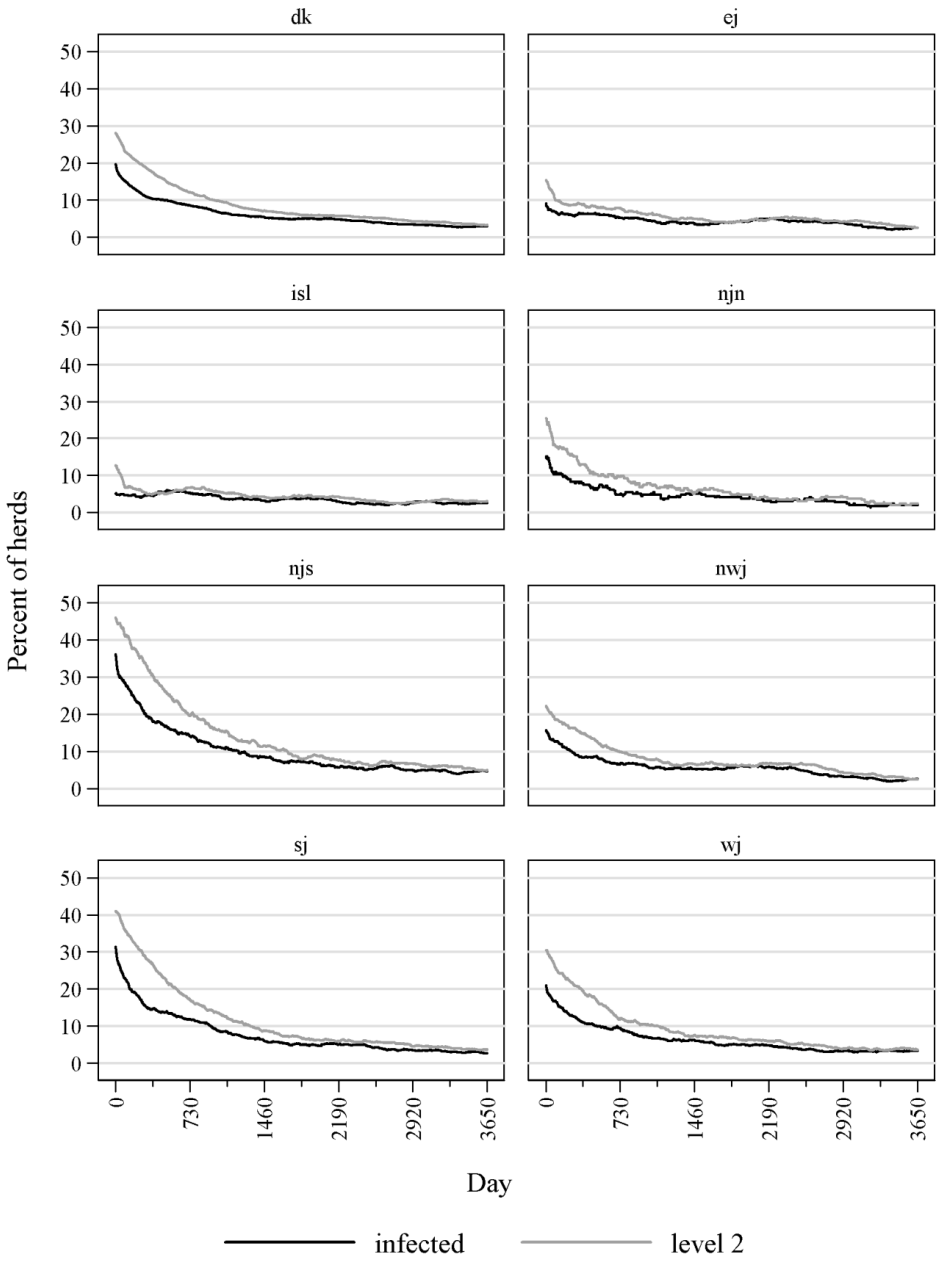

Figure 6 Predicted prevalence of cattle herds infected with *S*. Dublin and prevalence of herds classified as Level 2 (high risk) under Scenario 1 from a single iteration of 3650 days duration (ten years). Predictions are provided for all of Denmark (dk) and each of seven regions (ej, isl, njn, njs, nwj, sj and wj).

There is initially substantial variation between regions for both outcomes but the inter-regional variation diminishes with time. It appears that once true prevalence of herds infected with *S.* Dublin falls below about 10% (from 1 to 3 years depending on region) further reductions are

gradual. The prevalence of Level 2 herds is almost always greater than the true prevalence and the reduction in prevalence of Level 2 'lags' the fall in herd true prevalence. The predictions at t=3650 days from 1000 iterations of the base scenario are presented in Figures 7 and 8 (provided for comparison with the other scenarios). At t =3650 days there is a national median of 3.25% of herds infected and a median 4 % are classified as Level 2.

Comparison of scenarios

Output for the simulation related to restricted regional trading (Scenario 2, national median herd prevalence after 10 years of 3.38%) were derived assuming herds can only acquire replacement cattle from other herds located in the same region. Compared to the base scenario (Scenario 1) this restricting geographic movement of cattle delivers a dramatic benefit to those regions that have an initial low prevalence of Level 2 herds (especially EJ, ISL but also NJN and NWJ to a lesser extent). However, a penalty for the gains made at year 10 by these initially 'low prevalence' regions is that the remaining regions (NJS, SJ and WJ) have a higher prevalence of infection (and Level 2) than is the case under free trading.

The various measures used to mimic enhanced biosecurity (Scenario 3: less frequent trading of cattle, smaller consignments of cattle during trading, and less high risk trading) were predicted to have a dramatic impact on control of *S*. Dublin in Danish dairy herds. For example, the national (median) herd prevalence at 10 years is predicted to be 0.1% (compared to 3.25% in base scenario) and that of the regions more than a tenfold reduction compared to the base scenario.

Although increasing the frequency of testing to once in 30 days (Scenario 4) does improve the predicted outcomes at 10 years (national median herd prevalence at 10 years of 1.55%), the amount of this improvement is much smaller than obtained with enhanced biosecurity (Scenario 3) and enhanced control within infected herds (Scenario 5, national median herd prevalence at 10 years of 0.18%). Scenario 5 does suggest a very pronounced benefit if herds that become Level 2 can rapidly eliminate infection from their animals. Under this scenario, the median national prevalence of infection was 0.01% after 10 years, this being much lower for all other scenarios evaluated. Although the output from the composite strategy shows the greatest improvement over the base scenario compared to all other scenarios (national median herd prevalence at 10 years of 0%), both prevalence of infected herds and prevalence of Level 2 herds for the composite strategy are only marginally lower than those for the enhanced biosecurity scenario (Scenario 3). These results indicate that the herd biosecurity component of the composite strategy had a dominant effect on the model predictions for the latter scenario.

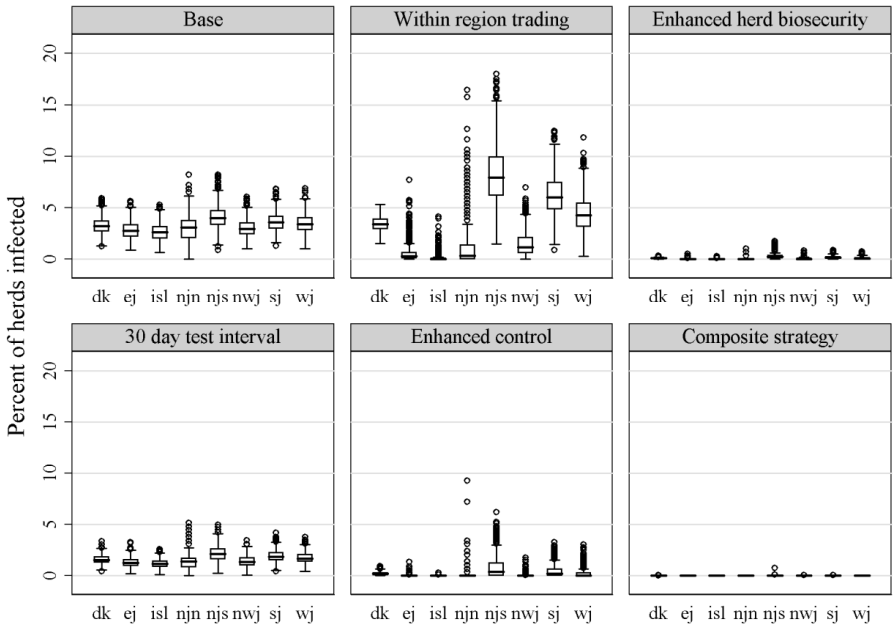

Figure 7 Simulation output (1000 iterations for each of six simulation scenarios) giving box plots of the predicted prevalence of herds infected with *S*. Dublin after 10 years of control. Results are provided for all of Denmark (dk) and each of seven regions (ej, isl, njn,njs, nwj, sj and wj).

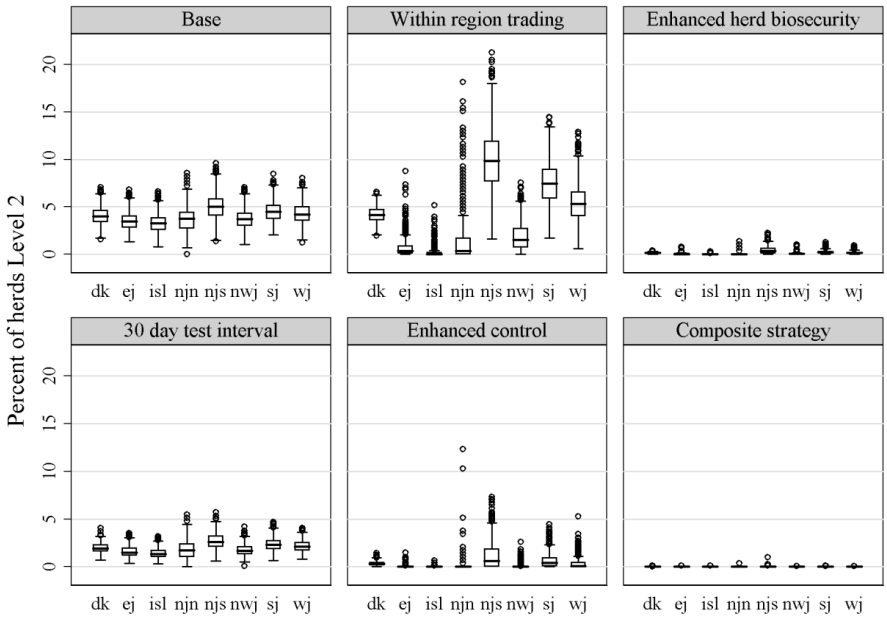

Figure 8 Simulation output (1000 iterations for each of six simulation scenarios) giving box plots of the predicted prevalence of herds classified as Level 2 (high risk) within the *S*. Dublin surveillance system after 10 years of control. Results are provided for all of Denmark (dk) and each of seven regions (ej, isl, njn,njs, nwj, sj and wj).

Discussion

We have demonstrated how a virtual hierarchy of objects can be useful for predicting the spread of infection in populations in the presence of surveillance and intervention programs of varying complexity. This approach is a major departure from traditional methods for modelling diseases as it explicitly simulates the infection and surveillance status of each individual element at each level of the hierarchy instead of dealing with elements *en masse*. By dealing with individual objects in computer memory it is possible to assign them any number of attributes for modelling the course of disease and the impact of interventions. Although, this approach to modelling disease is highly extensible, the degree to which this advantage can be exploited is limited by the extent of knowledge and data available from the population in question. Fortunately there is an extensive body of information in the DCD and from earlier studies on *S*. Dublin in Denmark that were extremely useful for informing the development of the present model. By using a hierarchical structure to manage information in the model we avoided the complexity that arises with other programming techniques and which have previously discouraged the development of similar models. Aside from providing a natural representation of the population, the hierarchy approach yields a specific advantage of being able to estimate differences in *S*. Dublin herd prevalence between regions and through time.

In practical terms this study has highlighted opportunities for hastening the elimination of *S*. Dublin from the Danish dairy industry. The model predicts that decisive progress is possible if the amount of time that herds are infected can be reduced and if biosecurity with regard to trade of animals can be improved. In contrast, more frequent testing of BTM for antibody to *S.* Dublin promises far less gain. There is also a strong indication that future control should be tailored to suit particular regions given the predicted disparity in prevalence estimates between regions even after many years of a control program. For example, region-specific programs could target aspects of herd biosecurity since the effect of these practices as assessed (Scenario 3) did have a strong influence at reducing herd-level prevalence (compared to Scenario 1) but might not be able to be implemented on a nation-wide scale for because it would demand too many resources. Herd-level biosecurity could also be combined with 'regional biosecurity' where herd managers within low prevalence regions are encouraged to only acquire replacement animals from low prevalence regions. Comparison of the output for Scenario 1 (base scenario) and Scenario 2 (restricted regional movement) suggests that some such form of 'regional biosecurity' would do much to protect the progress already made with the control of *S*. Dublin in low-prevalence regions.

The measures adopted in national disease control programs are usually arrived at after a range of interest groups make a joint consideration of scientific, practical, economic and social factors. For this reason it is presently difficult to suggest which particular combination of the scenarios that we have evaluated should be implemented despite our results demonstrating that some approaches have clear advantages over others. Useful comparisons of the economic consequences of different approaches to control of *S.* Dublin are available for the dairy industry in the Netherlands (Bergevoet et al., 2006), but may not be directly relevant to Denmark. Moreover, further work is needed on feasibility and affordability of the measures identified here as useful. For example, the extent to which herds can be more rapidly cleared of infection by reducing the spread of pathogen within Level 2 herds is not well quantified nor is it clear what resources would be required to achieve this. Nevertheless, while such information is being sought, the model can still be used to address decision options. We envisage this would involve combining the output of this study, with

the findings from additional scenarios arrived at during consultation with stakeholders. The model has a modern software interface so that any recommended strategies that emerge from this process can be interactively demonstrated to interest groups in the process of finalising research priorities and policy directions.

Ignorance about the ecology of *S.* Dublin as it occurs outside of bovine hosts dictates that there is much uncertainty in the way we modelled transmission of this pathogen between herds by environmental pathways. It is clear from the results in Figure 2 that the manner and amount of environmental transmission occurring in nature is critically important, both in a practical setting for preventing new outbreaks and with respect to the interpretation of output from the present model. Although we use a constant rate of transmission through environmental pathways this is less intuitively appealing than having the risk of environmental transmission made a function of regional prevalence of infected herds or a function of prevalence of infected herds in the immediate geographic vicinity of each individual herds. A greater understanding of environmental transfer of *S.* Dublin between herds is therefore of pressing importance. However, obtaining quantitative descriptions of the environmental transfer of *S*. Dublin will probably require a new development in methodology. Analysis of risk factors is a quantitative approach that has been used to examine aspects of environmental transfer in the past (Nielsen et al., 2007), but the outputs from this methodology are in the form of a coarse measurement of association and so are poorly suited for use in a simulation model.

Other caveats apply to the findings from this work. We used a range of input variables most of which stay fixed as the model steps through time and this may not always be appropriate. For example, we did not model changes in the size and number of dairy herds despite likelihood that this will happen during the present period of restructuring in the Danish dairy industry. We did not change the duration of various intervals in the infection-recovery cycle with time, nor did we alter patterns of trading of live cattle with time, nor did we change the within-herd prevalence of infection with time. To include such relationships in the model would have amounted to substantial speculation due to the sparsity of information on these subjects.

Although the virtual hierarchy approach was very suited to this work it may be less useful when simulations involve very large population (millions) due to the demands on computer memory and processing speed. Despite these shortfalls we consider that the general approach of a virtual model and the specific example involving *S*. Dublin in dairy cattle does offer a transparent and objective alternative to other decision making processes that could be applied in the present setting.

In summary, we have demonstrated a virtual hierarchy model for improving the basis of decisions aimed at controlling pathogens in populations of herds. The example of *S*. Dublin in cattle in Denmark was shown to be well suited to this approach because of the extensive amount of surveillance data and supporting studies available. Model outputs predict that the future approach for control of *S*. Dublin in the Danish cattle industry could be based a combination of enhanced herd-level controls once new infections are detected, improved animal trading practices and regional biosecurity measures.

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PAPER XII

Salmonella Dublin faecal excretion probabilities in cattle with different temporal antibody profiles in 14 endemically infected dairy herds

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Salmonella Dublin faecal excretion probabilities in cattle with different temporal antibody profiles in 14 endemically infected dairy herds

Liza Rosenbaum Nielsen

Summary

This longitudinal field study investigated the hypothesis that persistently high antibody levels indicate a high risk of *Salmonella* Dublin shedding in animals in 14 endemically infected dairy herds. A hierarchical multivariable logistic regression was performed on 6,614 paired faecal cultures and four types of temporal antibody profiles from cattle ≥180 days old. Age and repeated measurements on animals nested within herds were taken into account. Overall, the prevalence of faecal shedders was low (0.3% and 2.8% in the lowest and highest risk groups, respectively). An important predictor of faecal shedding was young age. There was a significant, but modest increase in risk in cattle with persistently high or recently increased antibody levels, but no difference between these two groups. Contrary to previously recommendations, the detection of carriers by the use of repeated antibody testing is therefore not likely to be a plausible control option in most *Salmonella* Dublin infected dairy herds.

Introduction

Salmonella enterica subsp. e*nterica* serovar Dublin (*S*. Dublin) is a gastrointestinal bacterial infection prevalent in many cattle herds worldwide. It causes increased morbidity, mortality and production losses (Richardson and Watson, 1971; Nielsen et al., 2010; Nielsen et al., 2012b). Even though it is host-adapted, it occasionally causes human infections that tend to be severe due to the invasive nature of this infection (Jones et al., 2008).

Controlling *S*. Dublin in cattle herds requires intervention to minimize the exposure to bacteria in the environment or shed by other animals in the herd (Boqvist and Vågsholm, 2005; Nielsen and Nielsen, 2012). A test-and-cull strategy to remove persistently infected cattle has long been considered an important control element (Richardson, 1973; Smith et al., 1992; Nielsen and Vestergaard, 1992; House et al., 1993). However, this recommendation is mainly based on limited, potentially biased study materials or experimentally induced infections (Spier et al., 1990; Spier et al., 1991; House et al., 1993). If a test-strategy involving repeated antibody testing of all or groups of cattle in infected herds is implemented as part of a control programme, and previous recommendations concerning the interpretation of the obtained antibody profiles for each individual animal are used (Spier et al., 1990; Smith et al., 1992; Nielsen et al., 2004a), it may lead to a long list of heifers and cows suspected as carriers of *S*. Dublin, in particular under high prevalence conditions (Nielsen and Dohoo, 2011). It will often not be economically feasible for the farmer to cull that many animals. Furthermore, there were indications in previous studies that not all of the suspected carrier animals actually pose a risk in the herd (Hoorfar et al., 1996; Lomborg et al., 2007). Hence, there is a need to quantify the risk posed by cattle with different temporal antibody profiles to facilitate prioritisation of risk management or culling decisions in the control of *S*. Dublin.

The objective of this study was to investigate the hypothesis that cattle with persistently high antibody levels are at higher risk of shedding *S*. Dublin through faeces than cattle with recent increases, fluctuating or moderately high antibody level, or low antibody levels. This study was

focused on *S*. Dublin for two reasons: (1) *S*. Dublin is the most commonly isolated serotype in Danish cattle, and the same is true for several other countries; (2) detection of persistently infected carriers by use of serology is to the author's knowledge only used for control of *S*. Dublin.

Materials and methods

Selection of herds and sampling

In 2000, a total of 14 dairy herds in the southern part of the peninsula Jutland of Denmark were selected to participate in a field study based on having bulk-tank milk *S*. Dublin ELISA results > 50 ODC% (background corrected optical density values (Nielsen and Ersbøll, 2005). At that time around 25% of the approximately 9000 Danish dairy herds had bulk-tank milk *S*. Dublin ELISA values > 50 ODC%. Herd size of the 14 selected herds was between 15 and 121 lactating cows (and between 69 and 262 animals in total) across all herd visits. Eleven of the herds mainly consisted of the Danish Holstein breed and 3 mainly consisted of the Jersey breed. Management, housing system and feeding practices were not recorded, but were likely to be similar to other *S*. Dublin infected herds in Denmark at the time.

S. Dublin was isolated from faecal samples at least once from these herds during the study period from beginning of 2000 to beginning of 2002, and there were indications of the herds being endemically infected throughout the project period (i.e. continued serological responses in all age-groups of cattle, or faecal or environmental samples being culture positive). All except one of the 14 herds were visited five times with approximately 3 months between each visit; the last herd was visited four times. At each visit, blood samples were collected from all calves, young stock and dry cows on the premises, and milk samples were collected from all lactating cows at the morning milking, for serological analysis. Faecal samples were collected rectally from all accessible animals into marked faecal sample containers aiming at getting at least 50 g from each animal. The samples were transported directly to the Danish Cattle Health Laboratory (DCHL) in Ladelund, and kept below 5°C until they were analysed within a few days after their arrival. At the laboratory, the faecal samples were pooled five at a time using 5 g per sample which was mixed to a 25 g pool before the analysis.

Laboratory analyses

Pooled faecal samples were examined at DCHL for the presence of *Salmonella* bacteria using standard procedures described elsewhere (Nielsen and Ersbøll, 2004; Nielsen et al., 2011). If the pool was found positive for *Salmonella* the individual samples were cultured using 25 g of faecal material to try to locate those animals that were positive in the pool. It has been estimated that using the pooling procedure may lower the sensitivity of the culture method to approximately half of the sensitivity of the method using individual samples in the first step (Nielsen, 2003). Serotyping and confirmation of positive isolates were conducted at the Danish Veterinary Institute in Copenhagen (today the National Food Institute, Technical University of Denmark). Whereas the analytic sensitivity of the test in the laboratory is reasonably high, i.e. approximately 80% in samples with 10 CFU/gram faeces (Baggesen et al., 2007), the diagnostic sensitivity for detection of infected cattle in naturally infected herds has been estimated to be very low, approximately 6-14% in subclinically infected cattle (Nielsen et al., 2004).

The *S.* Dublin ELISA used in this study was performed at DCHL slightly modified from a previously described ELISA method (Hoorfar et al., 1994). An O-antigen based *Salmonella* serogroup-D

lipopolysaccharide (LPS) preparation produced at the Danish Veterinary Institute in Copenhagen was used in the assay. This means that the ELISA mainly targets *S*. Dublin in cattle. However, cross-reactions with other serovars that share O-antigens with *S*. Dublin may occur (Konrad et al., 1994). The laboratory procedure was described in detail by Nielsen and Ersbøll (2004). An ODC%-value, which is a background-corrected proportion of the test sample optic density (OD) to the positive reference samples, was calculated as follows:

$$ODC\% = \frac{\left(\overline{OD}_{sample} - \overline{OD}_{neg\ ref}\right)}{\left(\overline{OD}_{pos\ ref} - \overline{OD}_{neg\ ref}\right)} * 100\%$$

where $\overline{OD}_{sample}$ is the average value of two test wells, $\overline{OD}_{neg\ ref}$ and $\overline{OD}_{pos\ ref}$ are the average values of four reference wells in the ELISA plates. The ODC% values were used to categorise cattle into antibody profile groups as described below.

Definition of antibody profile groups in individual cattle

ELISA results from animals aged <90 days were discarded before categorisation of cattle, because the diagnostic sensitivity and specificity of the test are known to be compromised by impaired capability of antibody production in calves aged <11-12 weeks (Da Roden et al., 1992) and maternally derived antibodies from colostrum (Nielsen, 2003). The final dataset contained the 3097 animals that were tested at least twice in the study herds. A total of 335 animals (9.8%) were not included due to lack of sufficient samples. The ELISA results from the animals that were aged ≥180 days at each herd visiting date were used to group the animals into four temporal antibody profiles (TAP) on each of the last four sample dates in the herds. The categorisation explanations, criteria and distribution of animals and faecal positive animals in each TAP category are provided in Table 1. Using these definitions, an animal that was only sampled once could not be included in the dataset. The age on the visiting date was recorded. The age distribution in the TAP categories is also provided in Table 1. Thus, with about three months between each sample date, the definition of the TAP categories was based on up to 1 year's samples from the animals.

Statistical analyses

SAS® version 9.2 was used for the data management, descriptive and statistical analyses. A hierarchical multivariable logistic regression analysis was used to statistically compare the effects of TAP categories and age of the animals, and to predict the probability of faecal excretion of *S*. Dublin. The model took into account repeated measurements at animal level nested within herd using generalised estimating equations (GEE) using a REPEATED statement in PROC GENMOD in SAS. A significance level of 5% was used to evaluate the statistical evidence of the effect of the predictors. The interaction between age and the TAP categories was likewise tested at 5% significance level.

Results

There were a total of 6,614 observations representing 3,097 animals aged ≥180 days in the dataset for analysis. This left 1,750 observations that did not fit into any of the TAP categories, because there were too few samples on the animals, out of the dataset for analysis.

S. Dublin bacteria (or in three cases non-typable *Salmonellae*) were isolated in 46 (0.7%) of the 6,614 observations and 14 (0.8%) of the 1,750 observations that were not included in the analysis. Eleven

of these 14 isolates were found in cattle with ELISA results ≥50 ODC%, and the last three had ODC%= 24, 29 and 37, respectively. Eight of the 14 were aged < 1 year.

Table 1 shows the distribution of animals, observations, faecal culture-positive observations and age within each of the TAPs. Despite the fact that the percentage of faecal culture-positive animals was highest in the TAP1 and TAP2 categories, the absolute number of *S*. Dublin shedding cattle was to be found highest among animals with fluctuating or moderately high antibody levels. Figure 1 shows the distribution of observations in the four TAP categories in the study herds.

The parameter estimates, odds ratios and P-values from the final multilevel, multivariable logistic regression model are shown in Table 2. Increasing age was clearly associated with decreasing probability of faecal shedding (Fig. 2). Furthermore, differences in faecal shedding probabilities were found to differ significantly between TAP1 and TAP4, but no statistical difference was found between TAP1 and TAP2 or TAP3. TAP2 on the other hand had significantly higher probability of faecal shedding of *S*. Dublin than both TAP3 and TAP4.

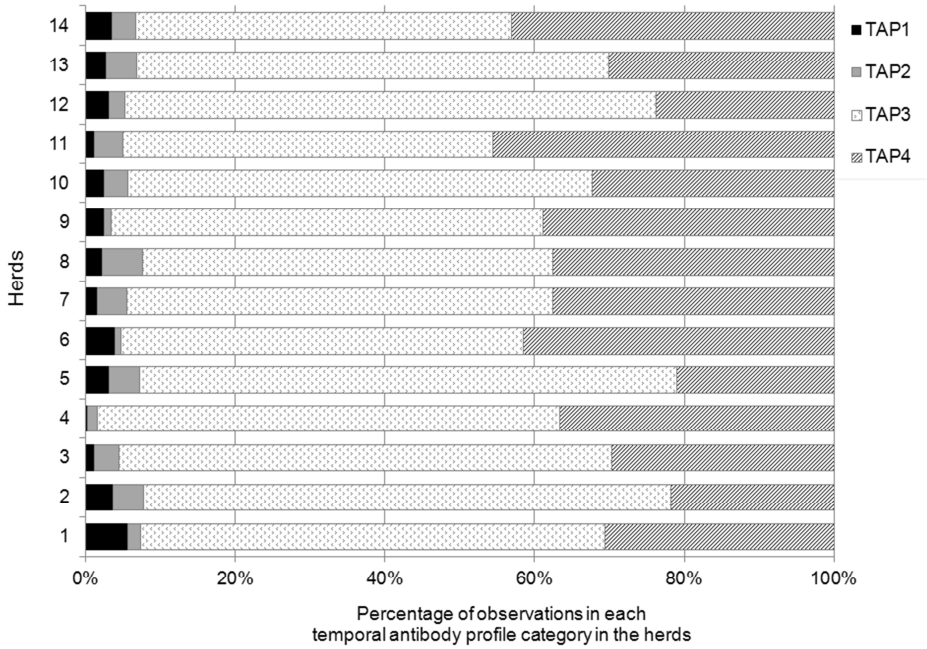


Figure 1 Distribution of *S*. Dublin temporal antibody profiles (TAP) in 14 Danish dairy herds. TAP1: Persistently high antibody levels, TAP2: Recently increased antibody levels, TAP3: Fluctuating or moderately high antibody levels and TAP4: Recently low antibody levels.

The parameter estimates, odds ratios and P-values from the final multilevel, multivariable logistic regression model are shown in Table 2. Increasing age was clearly associated with decreasing probability of faecal shedding. Furthermore, differences in faecal shedding probabilities were found to differ significantly between TAP1 and TAP4, but no statistical difference was found between TAP1 and TAP2 or TAP3. TAP2 on the other hand had significantly higher probability of faecal shedding of *S*. Dublin than both TAP3 and TAP4.

Discussion

In this study, a large field data collection from 14 endemically infected dairy herds was used investigated the hypothesis that cattle with persistently high antibody levels are at high risk of shedding *S.* Dublin and therefore are candidates to be culled or at least managed so that they will not spread the infection to herd mates. Despite that fact that there were seropositive animals in many of the age groups at most of the herd visits indicative of the herds being endemically infected, the general probability of shedding was very low (on average 0.7%) in all groups of cattle above 180 days old; only 46 samples out of 6,614 were found culture positive for *S*. Dublin. Apart from *S*. Dublin, only non-typable strains were isolated in 3 samples from the faecal cultures from 3 of the 14 herds. These were thought very likely to be *S*. Dublin by the Danish Veterinary Institute in Copenhagen (personal communication with Dorte Lau Baggesen), and were therefore included as such.

Based on this study material there was no evidence that animals with persistently high antibodies over a time period of at least half a year were at higher risk of shedding *S*. Dublin bacteria in faeces than other seropositive cattle. The only cattle that had significantly lower probability of faecal positive cultures than the rest of the TAP-categories were those with persistently low antibody levels (TAP4). In general the proportion of observations in the TAP1 and TAP2-categories were low compared to TAP3 and TAP4 in these 14 dairy herds. However, some of the TAP3 observations were based on two consecutively high antibody measurements ≥80 ODC%, which could not yet be categorised as persistently high until one more sample was available.

TAP1 and TAP2 could not be assigned to the same animal more than twice, whereas the other two categories could be assigned to the same animal up to three times. That was, however, not the explanation for the large differences in proportions of observations in the TAP-categories. The highest number of TAP1 observations observed in one herd was 25 (3% out of 897 observations in herd 13) and the highest proportion of TAP1-observations within a herd was 6% (19 observations out of 334 in herd 1). These numbers indicate how many suspected carriers there were present in the herds at most at any given point in time and which should be considered for culling, if the recommendations from previous studies were to be followed in a control scenario (Smith et al., 1989; Smith et al., 1992).

TAP2 had significantly higher probability of faecal shedding of *S*. Dublin than both TAP3 and TAP4. This group was also generally younger than the other groups, but since age was accounted for in the model, this was not the only explanation. A recent increase in the antibody levels in the TAP2-category was suggestive of recent exposure, which also increases the risk that the animal is still infected and may be excreting bacteria (Robertsson, 1984; Nielsen et al., 2007).

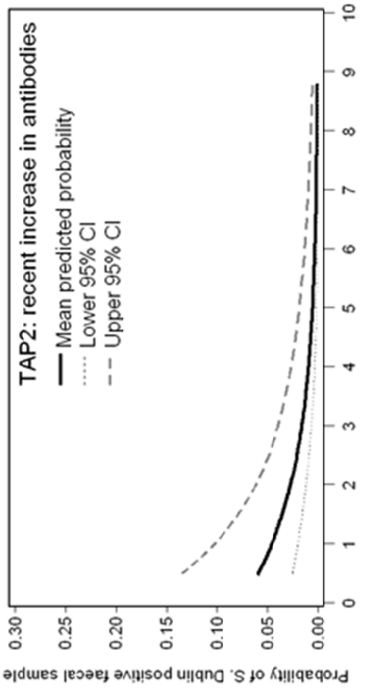
The animals in the TAP3-category also had significantly higher probability of faecal excretion than the TAP4-category, and because it was the biggest group of cattle, this was the group excreting bacteria most frequently in absolute numbers. The group consisted of cattle with fluctuating or continuously moderately high antibody measurements. The most likely explanation for such temporal antibody profiles is that the animals have been exposed repeatedly over time from herd mates or the contaminated environment. This would probably mainly include exposure to much smaller doses of bacteria than under experimental infection trials, which in turn may lead to lower or slower immune responses together with few clinical signs (Wray and Sojka, 1981; Robertsson, 1984).

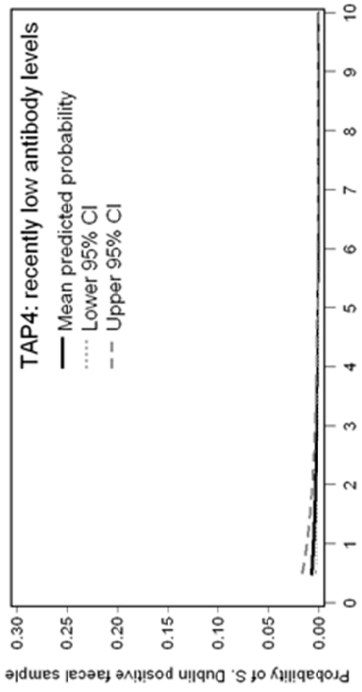
Table 1 Temporal antibody profile (TAP) criteria and distributions of animals, observations, faecal culture positive observations and age in 14 dairy herds that were endemically infected with *S.* Dublin in Denmark between 2000 and 2002. The TAPs were based on the 2 or 3 most recent samples from each animal ≥180 days old. The TAP groups were mutually exclusive.

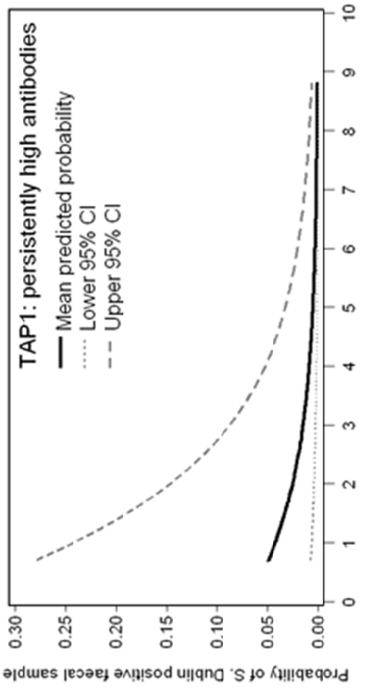
Group and short explanation	Criteria	No. of animals[a]	No. of (%) obs	No. of (%) FC pos[b]	Average age in years (95% CI)
TAP1 – Persistently high antibody levels	The current and the previous 2 samples ≥80 ODC%	126	182 (2.8%)	3 (1.7%)	3.8 (3.6-4.1)
TAP2 - Recent increase in antibody levels	The current sample ≥50 ODC% and the previous 1 or 2 samples <25 ODC%	214	214 (3.2%)	6 (2.8%)	2.7 (2.4-2.9)
TAP3 - Fluctuating or moderately high antibodies	One of the previous samples ≥25 ODC%, but not 3 samples ≥80 ODC%	1696	4022 (61%)	31 (0.8%)	3.4 (3.3-3.4)
TAP4 – Recently low antibody levels	The current and the previous 1 or 2 samples <25 ODC%	1061	2196 (33%)	6 (0.3%)	2.9 (2.8-3.0)

[a] Number of animals represented in the number of observations

[b] Percentage of the sample event observations in which *S.* Dublin bacteria were isolated


TAP2: recent increase in antibodies
Mean predicted probability
Lower 95% CI
Upper 95% CI
Probability of S. Dublin positive faecal sample
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0 1 2 3 4 5 6 7 8 9 10
Age in years


TAP4: recently low antibody levels
Mean predicted probability
Lower 95% CI
Upper 95% CI
Probability of S. Dublin positive faecal sample
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0 1 2 3 4 5 6 7 8 9 10
Age in years


TAP1: persistently high antibodies
Mean predicted probability
Lower 95% CI
Upper 95% CI
Probability of S. Dublin positive faecal sample
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0 1 2 3 4 5 6 7 8 9 10
Age in years

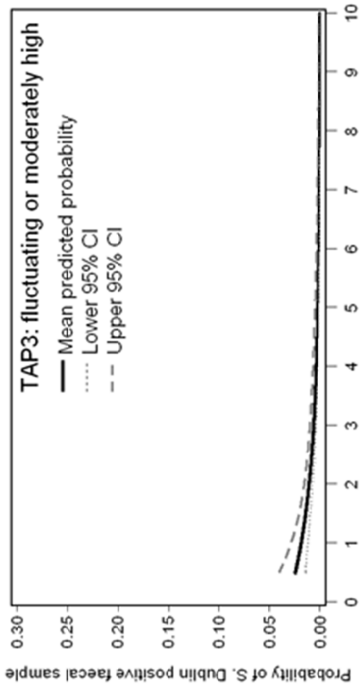

TAP3: fluctuating or moderately high
Mean predicted probability
Lower 95% CI
Upper 95% CI
Probability of S. Dublin positive faecal sample
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0 1 2 3 4 5 6 7 8 9 10
Age in years

Table 2 Final hierarchical multivariable logistic regression model of predictors for *S*. Dublin isolation from faecal cultures in 14 endemically infected dairy herds

Predictors	*β*	S.E.	OR (95%CI)	*P*
Intercept	-4.680	0.477		
Age in years	-0.544	0.136	0.6 (0.4-0.8)	<0.0001
Temporal antibody profile (TAP)				0.002
TAP1: Persistently high antibodies[a,b]	2.103	1.056	8.2 (1-65)	
TAP2: Recent increase[a]	2.183	0.585	8.9 (3-28)	
TAP3: Fluctuating or moderately high antibodies[b]	1.258	0.440	3.5 (1-8)	
TAP4: Recently low antibodies[c]	0	-		

OR, Odds ration; CI, confidence interval

[a,b,c] Variable levels with different subscript letters were significantly different at 5% significance level.

The results from the modelling of faecal excretion provided quite uncertain parameter estimates due to the low number of faecal positive cultures. This resulted in very wide confidence intervals for the odds ratios, which should therefore be interpreted with care. This calls for some reflection about how to quantify the risk associated with individual animals in endemically infected herds. One might argue that 14 dairy herds is not a sufficiently large sample of herds, and that some endemically infected herds might have a higher prevalence levels of faecal shedders than these study herds did. Previous studies suggest that this is not the case (House et al., 1993; Veling et al., 2002). Rather than selecting animals with persistently high antibodies to follow and see if they would shed bacteria, like it was done in the study of carriers by House et al. (1993), the present study was based on repeated paired samples on all cattle present in the farm over a 1-year period, and therefore had the potential to provide much less biased results in the evaluation of differences between TAP-categories. However, such a sampling frame is very time-consuming and expensive, so adding more herds is not a very obvious choice under economical limitations. One way to construct a cheaper sampling frame would be to perform repeated serology, which is the cheaper of the two laboratory procedures, e.g. 3 times and then collect faecal samples from the tested animals with 3 ELISA results available or a stratified random sample of these.

Furthermore, the faecal culture test used in this study is known to have poor diagnostic sensitivity (~6-14%) for detection of infected cattle (Nielsen et al., 2004), but these may not necessarily be shedding bacteria. It probably has better sensitivity (~80%) for detection of infectious animals (i.e. faecal shedders) (Baggesen et al., 2007). However, access methods with improved sensitivity for detection of bacterial shedding would be useful for research studies of potentially persistently infected carriers of *S*. Dublin.

Regardless of the limitations in sample size of faecal positive animals, there was a very clear association between age and faecal shedding probability in this study. The younger the animals the more likely they were to excrete *S*. Dublin. The highest estimated probability occurred in 180 days old calves. Here it was on average 5-6% in TAP1 and TAP2, around 2.5% in TAP3 and 1.5% in

TAP4, whereas cattle > 3 years old on average were faecal culture positive for *S*. Dublin less than 2% of the time regardless of the temporal antibody profile.

The implication of the study is that *S*. Dublin carrier detection based on repeated antibody measurements should be regarded as a very uncertain method to be used as a control element in persistently infected dairy herds. The age associations pointed to a more likely benefit of directing the focus towards methods to prevent spread of bacteria between calves and young stock, including consistent sectioning and careful cleaning of the environment and housing equipment on a regular basis as suggested in previous studies (Nielsen and Nielsen, 2012; Nielsen et al., 2012a; Nielsen et al., 2012b).

Acknowledgements

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PAPER XIII

Effects of experimental immunosuppression in cattle with persistently high antibody levels to *Salmonella* Dublin lipopolysaccharide O-antigens

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Effects of experimental immunosuppression in cattle with persistently high antibody levels to *Salmonella* Dublin lipopolysaccharide O-antigens

Sanne Ross Lomborg, Jørgen Steen Agerholm, Asger Lundorff Jensen and Liza Rosenbaum Nielsen

Abstract

Background

Salmonella Dublin (S. Dublin) is a zoonotic bacterium which is host adapted to cattle. The bacterium can cause subclinical persistent infection in cattle (carriers), which may be reactivated. During reactivation, animals may shed bacteria, thus constituting a source of infection for other animals. Identification of such carriers is assumed to be critical in attempts to control and eradicate the infection. Some authors suggest that persistently high antibody levels in serum or milk is indicative of a carrier state in cattle. However, this has been questioned by other studies in which S. Dublin were not found in all animals suspected of being carriers based on antibody measurements when such animals were examined at slaughter. Some hypothesize that the lack of isolated bacteria from long-term high antibody level cattle is due to a latent infection stage that can later be reactivated, for instance during stress around calving or due to transportation.

This study examined nine adult cattle with persistently high antibody responses to S. Dublin O-antigen based lipopolysaccharide for cultivable bacteria in faeces, milk and internal organs before and after transportation, isolation and experimental immunosuppression with dexamethasone sodium phosphate over a period of 7-14 days.

Results

Clear signs of immunosuppression were seen as expression of leucocytosis and neutrophilia in all animals on day 3-5 after the first injections with dexamethasone sodium phosphate. No clinical signs or necropsy findings indicating salmonellosis were observed in any of the animals. No shedding of S. Dublin was found in faeces (collected four times daily) or milk (collected twice daily) at any point in time during the 7-14 day period. S. Dublin was recovered by a conventional culture method from tissue samples from mammary lymph nodes, spleen and liver collected from three animals at necropsy.

Conclusions

In this study, immunosuppression by transportation stress or dexamethasone treatment did not lead to excretion of S. Dublin in milk or faeces from infected animals. The study questions the general conception that cattle with persistently high antibody levels against S. Dublin O-antigens in naturally infected herds should be considered high risk for transmission and therefore culled as part of effective intervention strategies. It is suggested that the location of S. Dublin infected foci in the animal plays a major role for the risk of excreting bacteria.

Background

Salmonella enterica subsp. *enterica* serovar Dublin (*S.* Dublin) is a zoonotic bacterium which is host adapted to cattle. Although it infects cattle at all ages, severe clinical disease is mostly seen in calves (Rings, 1985). The bacterium occasionally infects humans where it causes severe illness and high case mortality due to septicaemia (Helms et al., 2003).

An epidemiologically important feature of *S*. Dublin is its ability to cause subclinical persistent infection in cattle (carriers) (Richardson, 1973). Such carriers probably harbour the bacterium in cells of the reticular-endothelial system such as the liver and spleen (Lax et al., 1995) and it is assumed that reactivation of the infection can occur (Richardson, 1973; Grønstøl et al., 1974b; Counter and Gibson, 1980). It has been hypothesized that reactivation may be caused by stress due to transport or immunosuppression (McCaughey et al., 1971; Grønstøl et al., 1974b; Spier et al., 1991). During reactivation animals may shed bacteria and contaminate the environment, thus constituting a source of infection for other animals (Wray et al., 1989). Identification of such carriers is assumed to be critical in attempts to control and eradicate the infection (Spier et al., 1990; Smith et al., 1992; House et al., 1993; Veling et al., 2000).

Bacteriological culture is a common method to diagnose salmonellosis, but due to intermittent shedding of bacteria in milk and faeces by carrier animals, sensitivity of conventional bacteriological culturing is poor in such animals (House et al., 1993; Nielsen et al., 2004). However, serological analyses have indicated that carrier animals elicit a more persistent antibody response to *S*. Dublin lipopolysaccharide (LPS) than recently infected animals that have eliminated the infection (Smith et al., 1989; Spier et al., 1990; House et al., 1991; House et al., 1993). This has formed the basis for recommendations for control of *S*. Dublin, i.e. identifying carriers by demonstration of persistently high antibody levels against *S*. Dublin LPS by ELISA on blood or milk (Smith et al., 1992; Veling et al., 2000). The positive predictive value of the test is, however, questionable, meaning that not all animals detected as carriers based on antibodies are truly infected. It has been shown that the bacterium can be isolated at slaughter from around 50% of such persistently seropositive cattle (Hoorfar et al., 1996). A low positive predictive value has negative economic implications for the producers, because productive animals may be culled at disadvantageous times. On the other hand, a low negative predictive value would allow for undesired and unknown transmission of infection in the face of a test-and-cull strategy for handling of carrier animals.

Effective and cost efficient eradication of *S.* Dublin infections in cattle requires detailed knowledge about the pathogenesis of persistent *S.* Dublin infection, including risk assessment on animals with persistently high antibody titres, and the availability of tests with high predictive values for large scale screenings. The aim of this study was to evaluate if reactivation of a latent infection with *S.* Dublin occurs following transportation and immunosuppression in naturally infected cows with persistently high antibody responses to *S.* Dublin O-antigen based LPS. The study also adds further knowledge to the distribution of *S.* Dublin bacteria in tissues of cows with persistently high antibody responses.

Methods

Animals

Eight lactating Holstein cows (cases 1 - 7 and 9) and one Holstein heifer (case no. 8) (age range 1½ - 6 years, average: 4 years) of which three were pregnant were included in the study. The animals originated from four herds tested free for bovine virus diarrhoea virus and assumed to be free of several pathogens including bovine herpesvirus type 1, bovine leucosis virus, *Brucella abortus*, and *Mycobacterium bovis* due to the national disease status. The herds had naturally acquired infection with *S.* Dublin and participated in a *S.* Dublin intervention project. The animals were selected for the study based on antibody levels to *S.* Dublin LPS of at least 80 ODC % (background-corrected ratio of the optical density to a positive reference) during a period of at least 180 days measured by ELISA in three individual milk or blood samples collected every three months (Figure 1). While other cows in the herds had decreasing antibody levels these nine animals remained high in antibody levels for at least 180 days.

Experimental design

The animals were transported two at a time by truck for 4-6 hours from four herds of origin and were housed separately in isolation facilities at the research institution. The animals were separated by a wooden wall during transportation. The animals were examined clinically before they left the herd of origin and blood, milk and faeces were sampled (day 0). Additional blood, milk and faecal samples for serology, bacteriology or haematology were collected upon arrival at the isolation facility. Clinical examinations and sampling for bacteriological culture and serology were performed daily. The animals were treated with dexamethasone sodium phosphate (DSP) at a dose of 0.08 mg/kg intramuscularly (Dexadreson® Vet, Intervet International, Boxmeer, The Netherlands) on day 2, 3 and 4. The animals were euthanized by intravenous injection of a sodium pentobarbital solution seven to 14 days (average 7.8 days) after arrival and necropsied. Samples for bacteriology and histopathology were taken at necropsy. Case no. 9 was only included in a part of the study as it was euthanized on day one due to accidental injuries.

Housing and Management

The animals were housed separately in isolation facilities. Only two animals were allowed in the barn in the same period and they were housed alone in each of two fully closed isolation rooms. All daily routines were structured in a way to minimize the risk of cross contamination between two animals in adjacent isolation rooms. The animals were fed 4-6 kg concentrated feed twice daily and had free access to grass silage and water. Milking was done twice daily in lactating animals. The study was performed during a three-month-period with ambient outdoors temperature of 7 to 20^{o}C.

Clinical examination

Full clinical examination was performed daily, while rectal temperature, respiratory and pulse rates were recorded twice daily.

Bacteriology

Milk samples (50 ml) of lactating animals were collected aseptically to avoid faecal contamination from all functional quarters twice a day and pooled, while faeces (50 g) was sampled rectally four times daily. The samples were stored at 4^{o}C until analysis, which was started within 24 hours,

except for samples taken on Fridays and Saturdays, which were stored for around 72 and 48 hours before analysis, respectively.

Tissue samples were taken at necropsy. Whenever possible, 25 g of tissue was sampled. The tissues included tonsils, lung, tracheobronchial lymph nodes, spleen, liver, liver lymph nodes, gall bladder, ileum, colon, colon lymph node, gut associated lymphoid tissue of colon (colon tonsil), cecum, cecal lymph node, uterus including placentomes if present, mammary gland and mammary lymph nodes. Lung, liver, gall bladder and abomasal content of an aborted foetus were also examined. Instruments were disinfected in 96% ethanol between each tissue sample.

The samples were cultured by conventional culturing as previously reported (Nielsen and Ersbøll, 2004). In short, Buffered Peptone Water (BPW, Merck 1.07728) were added to 25 g sample to a dilution of 1:10 and incubated over night at 37˚C. From the pre-enriched buffer, inoculation was performed on two enrichment medias: a) 100 ml onto Modified Semi-solid Rappaport Vassiliadis (MSRV, Oxoid CM910); b) 1 ml in 9 ml selenite cysteine broth (SC, Merck 1.07709). Both media were incubated at 41.5°C for 24 hours. Culture negative plates were incubated for further 24 hrs. After enrichment, 10 ml from the broths and material from swarming zones of probably positive MSRV-plant were inoculated in parallel onto Brilliant Green Agar (BGA, Oxoid CM 329) and Xylose Lysine Deoxycholate agar (XLD, Oxoid CM469). These plates were incubated at 37°C for 24 hrs. Isolated strains were verified by serotyping according to the Kauffman-White scheme.

Serology

Daily blood samples were collected from the jugular or caudal vein in unstabilised vacutainer tubes. The samples were centrifuged at 2,000 *g* at 4°C for 10 minutes after coagulation, which occurred during five to 24 hours of storage at 4°C. Serum was aspirated and stored in cryotubes at –18˚C until analysis.

The level of antibodies to *S.* Dublin LPS was analysed at Steins Laboratory, Holstebro, Denmark as previously described (Nielsen and Ersbøll, 2004). In short, analyses were performed by an indirect O-antigen based LPS serum- or milk-ELISA. Results were measured in optical density (OD) by an ELISA plate reader. The observed OD was corrected for background OD using negative test sera and expressed as ODC%:

$$ODC\% = (OD_{sample} - OD_{neg\ ref}) / (OD_{pos\ ref} - OD_{neg\ ref}) \times 100\%$$

where OD_{sample} = mean OD of the two test sample wells, $OD_{neg\ ref}$ = mean OD of the four negative reference sample wells, and $OD_{pos\ ref}$ = mean OD of the four positive reference test sample wells.

Haematology

Daily blood samples were collected as for serology in 10 ml EDTA-stabilised vacutainers for haematological profiles. Analysis was performed within the same day except for samples taking during weekends, which were stored at 4°C until examination. The samples were analysed by automatic flow cytometry using an automated analyser (ADVIA120, Bayer) with species-specific software. Automatic differential cell counts of leucocytes were carried out and histograms were visually inspected as were blood smears to evaluate if automatic counting was correct. If doubt arose, manual differential cell counts were performed on 100 leukocytes.

Histopathology

The animals were necropsied and evaluated for gross lesions. Lesions were sampled, fixed in 10% neutral buffered formalin, and processed for microscopy by routine histological methods. Tissue sections were stained by haematoxylin and eosin.

Results and discussion

Antibody levels

Nine animals from four dairy herds were included in the study. The antibody levels (*S.* Dublin ODC%) at time of arrival were above 80 for 8 animals, while it was 78 for one cow (No. 2). The ODC% had either slightly decreased or had remained at approximately the same level through the preceding 240 days (Figure 1).

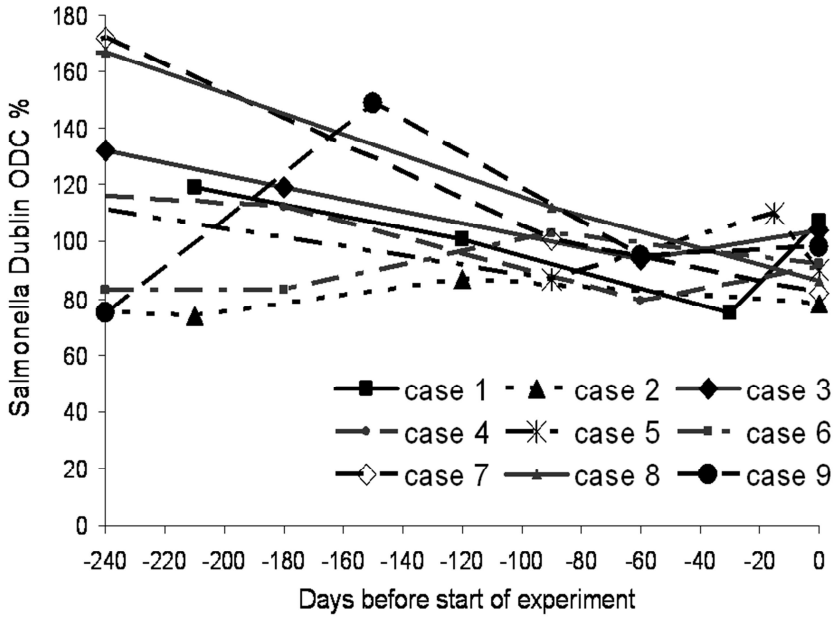

Figure 1 Repeated antibody measurements in nine cattle suspected as *S.* Dublin carrier animals at study start (day 0) and during the preceding 240 days. ODC% indicates the level of antibodies to *S.* Dublin O-antigen based LPS in serum or milk.

Clinical symptoms

The animals were of normal condition at arrival. During the study period mild symptoms related to localised *Sarcoptes* mange (No. 2), chronic mastitis or positive California Mastitis Test (Nos. 1-6) and traumatic injuries of the distal parts of the legs (No. 2) were observed. The most frequent abnormal clinical finding was reduced appetite (four cows). Furthermore, two cows had reduced rumen motility. Diarrhoea occurred in two animals (Nos. 4 and 8) after treatment with dexamethasone sodium phosphate (DSP). Cases Nos. 1 and 8 aborted on day 5.

Haematology

The haematological profiles were within the normal range from day 0 through 3, but a slight increase in the number of segmented neutrophilic granulocytes (SNG) occurred on day 0. On day 3 a marked increase in SNG due to leucocytosis and neutrophilia was observed (Figure 2).

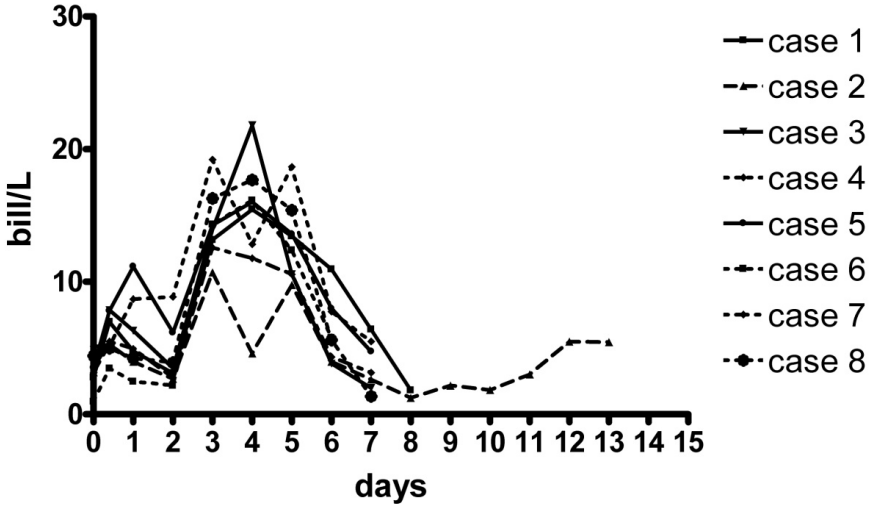

Figure 2 Measurements of segmented neutrophil granulocytes (SNG) in eight cattle with persistently high antibody levels to *S.* Dublin. The animals were injected with dexamethasone sodium phosphate on day 2, 3 and 4 (reference values: 0.60-5.65 billions SNG/L).

Necropsy

Necropsy revealed a range of lesions of which some were incidental findings as chronic multifocal interstitial nephritis (No. 3), and chronic mastitis (Nos. 3 and 6), while others were most likely related to the relocation of animals and DSP treatment (acute haemorrhagic abomasitis predominantly at the margins of the abomasal plicae (Nos. 1, 3, 4, 6, 7) and diffuse low-grade hepatic steathosis (No. 6)). Four cows had rumenitis probably due to rumenal acidosis before or during the study (Nos. 2, 3, 5, 9).

Bacteriological culture

Bacteriological culture of milk and faeces samples collected throughout the study failed to demonstrate any excretion of *Salmonella* sp. Bacteriological examination of 14-16 organ specimens from each animal sampled at necropsy revealed the presence of *S.* Dublin in three animals originating from one herd. *S.* Dublin was isolated from mammary lymph node and the liver of cows No. 3 and 7 respectively, while it was isolated from both the liver and spleen of No. 8. The aborted foetus of cow No. 8 was not examined. No other serotypes of *Salmonella* were found.

Cattle with persistently high antibody responses to *S.* Dublin LPS are expected to harbour the bacterium and control strategies for *S*. Dublin are often based on this hypothesis (Smith et al., 1989; Spier et al., 1990; Spier et al., 1991). We were only able to isolate *S.* Dublin from three out of eight cows and one heifer even though all fell within the detection criteria for carriers. This may be due to an insufficient sensitivity of bacteriological examination, but it may also reflect a complete elimination of bacteria by previously infected animals, in particular in adult cows. A previous study

in Danish cattle demonstrated *S.* Dublin in 2 of 14 (14%) persistently seropositive adult cows at necropsy despite extensive tissue culturing, while 10 of 17 (59%) of the young cattle (heifers and bulls) were culture positive at necropsy [18]. The animals all came from herds with recent clinical outbreaks of *S*. Dublin and no control strategies in place. In another study, 3 of 8 persistently seropositive adult cattle were culture positive at necropsy from animals that had *S.* Dublin isolated from both milk or faeces during the preceding six months indicating active infections prior to necropsy (House et al., 1993). They all came from the same large dairy herd which suffered from severe *Salmonella* Dublin related clinical problems and the herd used vaccination of cows as part of the control strategies. Thus, the study group in that study was not comparable to the cattle used in the present study. The persistent discrepancy between the serological and bacteriological findings may indicate that only few cows with long-term high antibody responses are truly infected with *S.* Dublin, thus making antibody based testing unreliable in adult cattle. The present study adds knowledge about persistently seropositive cows in herds that have performed control strategies for an extended period of time. These cows continued to be seropositive even under circumstances where herd mates had decreasing or low antibody titers to *S*. Dublin. It could be postulated that some cows with a strong antibody response in fact could be desirable, as they may reflect a superior capability to eliminate *S.* Dublin.

It is likely that excretion of bacteria in faeces and milk reflects the localisation of the infection in the gastrointestinal tract or the mammary gland, or a temporary infection from the environment. It is obvious that a surface lesion such as an intestinal mucosal ulceration is more likely to release bacteria to the environment than a lesion in internal organs such as the spleen. *S.* Dublin was not found in faeces of the cattle in this study, neither before nor after immunosuppression. In accordance with this, bacteria were not isolated from intestinal tissue or intestinal lymph nodes at necropsy. It is likely that the animals in this study did not have infectious foci in the intestinal tract. It is possible, that the culture negative results in faeces found in other studies may have a similar explanation and that discrepancy between culture positive faeces samples and culture negative intestinal tissue specimens may be due to passive transfer of orally acquired bacteria in the intestinal content without mucosal colonisation.

The detection of *S.* Dublin in the liver and spleen probably reflects a previous haematogenous spread. This may either have been restricted to the portal circulation with localisation only in the liver or have been systemic with localisation in multiple tissues. *S*. Dublin was only isolated from a mammary lymph node in one cow. This may reflect a previous *S.* Dublin associated mastitis, but it is more likely that the lymph node localisation is associated with a previous bacteraemia with a primary location in the lymph node or secondary to a localisation in the drainage area.

An important epidemiological aspect of bovine salmonellosis may be the reactivation and excretion of bacteria following stress and immune suppression (McCaughey et al., 1971; Grønstøl et al., 1974a; Grønstøl et al., 1974b; Spier et al., 1991). Reactivation apparently occurs rapidly. In an experimental study faecal excretion in carrier animals happened within one day after transportation (Grønstøl et al., 1974a; Grønstøl et al., 1974b). Therefore, a study period of 7 to 14 days was considered sufficient to detect reactivation if it occurred. The animals in this study were transported, relocated into isolation facilities and finally treated with DSP to induce immunodepression and subsequent reactivation of a latent infection. These events led to changes in the haematological profiles (Figure 2) consistent with DSP induced immune suppression, but

reactivation of the infection apparently did not occur. This may be explained by the location of bacteria in the actual animals combined with the mechanisms by which DSP induces immunosuppression. DSP induces a pronounced down regulation of surface L-selectin on circulating neutrophils, which impede their ability to adhere to the endothelium. Consequently, their migration into tissues is hindered and extravascular immunodepression develops (Weber et al., 2004). If it is assumed that bacteriological examination identified the only infected tissues (liver, spleen, and lymph node) then excretion of bacteria in milk or faeces would require haematogenous spread to the intestine and mammary gland. Thus, even if the bacteria were reactivated, they had to enter the blood to reach these organs. Since DSP does not impede the killing mechanisms of neutrophils (Hoeben et al., 1998; Burton et al., 2005), bacteria entering the blood probably would have been eliminated. The situation is likely to be different in animals harbouring *S.* Dublin in tissues with an external surface such as the intestine, tonsils and mammary gland. DSP treatment of such animals may reactivate the infection, and because haematogenous spread is not needed, such animals may release bacteria. This hypothesis is in accordance with the observations by Spier et al. (1991) who observed a significant increase in excretion of *S.* Dublin in milk of udder infected cattle following DSP treatment. In the experimental studies of the effect of transportation on shedding patterns in cattle, it is quite possible that the animals had bacteria in the gut even when they were not shedding before exposed to the stressors [5,9] therefore making it easy to return to a shedding state after transportation. Our study indicates that true reactivation of a latent infection located in organs outside external surfaces is not likely to occur under farm conditions.

Conclusion

This study raises several questions regarding the use of repeated antibody measurements for detection of cows persistently infected with *S.* Dublin and the pathogenesis of reactivation. The isolation of *S.* Dublin from only 2 of 8 adult animals despite intensive bacteriological culturing of both faecal matter, milk and target organs questions the reliability of both serology and bacteriology in this age group. It is important to have reliable diagnostic tools for identification of persistently infected animals to control the spread of the infection within and between herds efficiently, and the study emphasises these needs.

Though the sample size is not large, the study also indicates that the risk of excretion of bacteria may depend on the localisation of infectious foci. It is likely that cattle harbouring *S.* Dublin in organs without an external surface are of lower risk of releasing bacteria to the environment than animals with intestinal or mammary infections. Thus, reactivation and excretion of bacteria is probably not only a matter of immunosuppression and latent infection but mainly a question of tissue localisation.

Finally, it is possible that other mechanisms of immunosuppression different from those of DSP may lead to reactivation of latent infection, but the pathogenesis of such a mechanism in relation to *Salmonella* infection and reactivation remains to be described. In order to better understand the mechanisms, it appears to be important to differentiate between true latent infections and active persistent infections with continuous or intermittent shedding.

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PAPER XIV

Simulation model estimates of test accuracy and predictive values for the Danish *Salmonella* surveillance program in dairy herds

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Abstract

The Danish government and cattle industry instituted a *Salmonella* surveillance program in October 2002 to help reduce *Salmonella enterica* subsp. *enterica* serotype Dublin (*S*. Dublin) infections. All dairy herds are tested by measuring antibodies in bulk tank milk at 3-month intervals. The program is based on a well-established ELISA, but the overall test program accuracy and misclassification was not previously investigated. We developed a model to simulate repeated bulk tank milk antibody measurements for dairy herds conditional on true infection status. The distributions of bulk tank milk antibody measurements for infected and noninfected herds were determined from field study data. Herd infection was defined as having either ≥1 *Salmonella* culture-positive fecal sample or ≥5% within-herd prevalence based on antibody measurements in serum or milk from individual animals. No distinction was made between Dublin and other *Salmonella* serotypes which cross-react in the ELISA. The simulation model was used to estimate the accuracy of herd classification for true herd-level prevalence values ranging from 0.02 to 0.5. Test program sensitivity was 0.95 across the range of prevalence values evaluated. Specificity was inversely related to prevalence and ranged from 0.83 to 0.98. For a true herd-level infection prevalence of 15%, the estimate for specificity (Sp) was 0.96. Also at the 15% herd-level prevalence, approximately 99% of herds classified as negative in the program would be truly noninfected and 80% of herds classified as positive would be infected. The predictive values were consistent with the primary goal of the surveillance program which was to have confidence that herds classified negative would be free of *Salmonella* infection.

Introduction

Salmonella is an important pathogen in humans and domestic animals. In developed countries, most human cases of salmonellosis result from foodborne transmission from livestock sources. Human infections can also occur by direct contact with livestock (Hendriksen et al., 2004; Olsvik et al., 1985). In addition to the public health impact of zoonotic infections, livestock morbidity, mortality and the resulting economic losses are substantial for certain *Salmonella* strains. *Salmonella enterica* subsp. *enterica* serotype Dublin (*S*. Dublin) is a host-adapted serotype in cattle which occasionally infects other species (McDonough et al., 1999). Though relatively infrequently associated with human salmonellosis, *S.* Dublin may cause invasive infections in people resulting in serious illness and death. Among people hospitalized with salmonellosis, *S*. Dublin causes higher mortality than other serotypes (Helms et al., 2003). *S.* Dublin has received high priority in Danish cattle for several reasons. Human infections continue to occur in Denmark with between 25 and 45 human cases per year reported from 2001 to 2003 (Anon, 2004). Transmission to people may occur after consumption of improperly pasteurized milk products, but in Denmark the main source is probably insufficiently cooked meat (Fierer, 1983; Helms et al., 2003; Humphrey et al., 2000). In cattle herds, *S*. Dublin causes economic losses in the form of disease and death among calves and young animals, as well as abortions and reproductive disorders among adult cattle, extra farm labor and increased veterinary expenses (Hinton, 1974; Peters, 1985; Visser et al., 1997). Furthermore *S*. Dublin is the serotype isolated most frequently from cattle herds in Denmark (Anon, 2004).

In an effort to control *S*. Dublin in cattle, the Danish government and cattle industry initiated a national surveillance program in October 2002 (Anon, 2004; Pedersen, 2003). In this program, all cattle herds are classified into one of three *Salmonella* infection levels based on antibody measurements in bulk tank milk (BTM) or blood samples, fecal culture results and movement of animals between herds. For dairy herds, *Salmonella* program classification levels primarily are based on quarterly BTM ELISA measurements of *Salmonella* antibodies. Test results and animal movement data are recorded in the Danish Cattle Database. Level 1 is considered most likely to be free of *Salmonella*. At the time of this study, dairy herds were classified as Level 1 if both the average ODC% (background corrected optical density value from the ELISA) of the last 4 BTM samples was below 25 and no increase of more than 20 ODC% was found when comparing the most recent measurement to the average of the three previous measurements. Levels 2 and 3 were divided into two sub-levels. Level 2a included herds with BTM antibody measurements which exceeded either of the test criteria described above. Herds in Level 2b were those which were not classifiable due to lack of data or that had recorded contact with 2a or 2b herds. Such contact included purchase of cattle or contact via common pasture, markets, dealers or shows. Herds in Level 3a had clinical salmonellosis due to *S*. Dublin confirmed by bacteriological culture and Level 3b herds had the bacteria detected by culture, but clinical salmonellosis was not necessarily present, or the herd had contact with cattle from a Level 3a or 3b herd. The program was regulated by the Danish Veterinary and Food Administration and administered by the Danish Cattle Federation. Regulatory actions, including slaughter restrictions, were taken in Level 3 herds, but not in Level 2 herds. Program classification for all herds was publicly available on an internet site.

The purpose of this study was to estimate the accuracy and predictive values of the Danish *Salmonella* testing program for dairy herds. The analysis was performed only for classification of herds based on BTM *Salmonella* antibody measurements (Levels 1 and 2a) and did not consider classification based on other criteria which represented either very few herds (Levels 3a and 3b) or a regulatory status defined by trade or lack of data rather than based on test results (Level 2b).

Methods

A simulation model approach was used to estimate test program accuracy and misclassification. This was done in order to make efficient use of existing data. The BTM ELISA currently used in the surveillance program cannot distinguish *S*. Dublin infections from *S*. Typhimurium and possibly other serotypes (Konrad et al., 1994). Therefore, the definition of herd infection for this study included infection with *S*. Dublin, *S*. Typhimurium and other cross-reacting serotypes. We relied on data collected through the surveillance program and on results from a previous field study to estimate input parameters for the simulation model. The simulation study was carried out as follows:

Step 1: Field data were used to estimate parameters for the distributions of BTM antibody measurements and to estimate the correlation between repeated BTM antibody measurements from the same herd. These analyses were done separately for infected and noninfected herds with herd infection defined by individual animal *Salmonella* test results.

Step 2: BTM antibody distribution parameters and correlation coefficients from Step 1 were used to specify distributions for random simulation of quarterly antibody measurements

according to herd infection status over a 1-year period and then to calculate the probability of exceeding the surveillance program test cut-offs.

Step 3: Results from Step 2 were then used in a separate simulation model to estimate overall surveillance program test accuracy and predictive values at various levels of *Salmonella* infection prevalence among dairy herds.

The laboratory tests used in the field study and surveillance program, the surveillance program data and the specific modeling procedures are described in the following methods sections. Descriptive statistical analyses were performed using SAS ver. 9.1 (SAS Institute, Cary, NC) and simulations were performed using @Risk software (ver. 4.5.5, Palisade Corporation, Ithaca, NY 14850, USA).

Laboratory tests and field data (Step 1)

ELISA

The antibody ELISA used for both individual and BTM samples was similar to an indirect antibody ELISA described in detail elsewhere (Nielsen, 2003; Nielsen and Ersbøll, 2004). In short, microtitration plates were coated with a *Salmonella* Dublin lipopolysacharide antigen produced at the Danish Institute for Food and Veterinary Research (DFVF), where the test was originally developed (Hoorfar et al., 1995). Sample milk was added undiluted to the microtitration plate wells in duplicates. Known positive and negative reference milk was added in quadruplicates. Following incubation, bound immunoglobulins were detected by an affinity-purified horseradish peroxidase-labelled goat anti-bovine IgG (H + L) conjugate. Substrate and indicator solution were added and incubated in the dark for approximately 15 min. The reaction was stopped when the optical density (OD) of the positive reference wells was 1.5–2.0. An ODC% was calculated for each sample as follows:

$$ODC\% = \frac{\left(\overline{OD}_{sample} - \overline{OD}_{neg\ ref}\right)}{\left(\overline{OD}_{pos\ ref} - \overline{OD}_{neg\ ref}\right)} * 100\%$$

where $\overline{OD}_{sample}$ is the mean value of two test wells, $\overline{OD}_{neg\ ref}$ and $\overline{OD}_{pos\ ref}$ are the mean values of four negative and four positive reference wells in the ELISA plates.

Sensitivity (Se) and specificity (Sp) for the individual serum ELISA has been estimated (Nielsen and Ersbøll, 2004; Nielsen et al., 2004), but Se and Sp for the BTM test for diagnosing herd infection status was unknown.

Surveillance program data

BTM ELISA results, farm characteristics and official surveillance program classification for all Danish dairy herds were obtained from the Danish Cattle Database for the period from January 2001 to February 2004. Dairy herds were identified as herds with weekly bulk tank somatic cell counts recorded as part of a compulsory milk quality program. BTM ELISA data were used to assign quarterly classification levels to all dairy herds, calculate the proportion of Level 2a herds and evaluate the distribution of BTM ELISA measurements for herds with a recent history of *S*. Dublin isolation by bacterial culture. All ELISA ODC% below zero in the study database were set equal to

zero before further analysis. Level 2a classification was defined as having a four-measurement moving average of ≥25 ODC% or having a difference of >20 ODC% between the current measurement and the average of the previous three measurements. Most measurements were recorded at approximately quarterly intervals (94% of sampling intervals were from 2 to 4 months), but other test intervals occurred because of automatic retests and other special testing circumstances such as owner-requested tests. The probabilities for changing from Levels 1–2a and 2a–1 from one quarter to the next in the surveillance program data for seven geographic regions were calculated and used for comparison with simulation model calculations. The seven regions were created so that each contained approximately the same number of dairy cattle and included contiguous geographic areas which had similar *Salmonella* apparent prevalence in the surveillance program.

Field study data

Data from an earlier field study (Kongeå project) were used to estimate the distributions of BTM ELISA values for infected and noninfected herds. The data collection for the study was described in detail previously (Nielsen, 2003). In brief, samples for culture and serology were collected from 30 dairy herds that were visited up to 5 times at 3 months intervals during years 2000–2002. Each herd also had BTM *Salmonella* antibodies measured each month. The herds were selected based on BTM ELISA results in early 2000 with the goal of including farms with a wide range of within-herd *S*. Dublin prevalence. At each visit, the following samples were collected from cattle in confinement housing on the premises (pastured heifers were not tested at some visits during the grazing season): rectally collected fecal samples from all cattle, blood samples from all nonlactating cattle, and individual milk samples from all lactating cattle. Twenty swabs from the environment and dung pits were also collected. The fecal samples and swabs were examined by bacteriological culture to detect *Salmonella* bacteria at Steins Laboratory in Ladelund, Denmark. Positive samples were serotyped at DFVF. Blood and milk samples were analyzed for antibody levels at Steins Laboratory by the ELISA described above.

For the current study, Kongeå project herds were classified as infected at a particular sampling date if at least one individual animal was positive by fecal culture or the within-herd *Salmonella* apparent prevalence among tested cattle was ≥5%. For the prevalence calculations, an animal was considered *Salmonella* positive if it had an ELISA antibody measurement in serum or milk of ≥50 ODC%. Herds were classified as negative if
Salmonella was not isolated from any individual animal fecal sample and apparent prevalence was <5%. The fecal culture test and individual-animal ELISAs used for herd classification have been evaluated elsewhere (Nielsen and Ersbøll, 2004; Nielsen et al., 2004). BTM ELISA measurements taken within ±20 days of the within-herd prevalence estimate or sample collection for culture were used to describe the bulk-tank antibody distributions for infected and noninfected herd-visits.

Based on visual inspection of the data and on results from the @Risk distribution fitting procedure, we decided to use a normal distribution to model BTM antibody measurements from infected herds and an exponential distribution for noninfected herds. Parameters for these distributions were estimated from field study data. There were from 1 to 5 observations per herd with almost all herds contributing three or more observations. Therefore, random resampling was used to provide parameter estimates based on one observation per herd. This procedure was done

separately for infected and noninfected herd visits. One hundred samples were drawn with one measurement per herd selected at random for each sample. The number of observations per sample corresponded to the number of herds in the noninfected and infected categories. Two herds changed infection status during the field study period and contributed observations to both the infected and noninfected periods. BTM ELISA measurements from these two herds were included in the applicable category defined by their status at the time the sample was collected. For each of the 100 samples from the infected herd data, point estimates and 95% confidence intervals were calculated for the BTM antibody mean and standard deviation. For each of the 100 samples from the noninfected herd data, the mean BTM antibody and its 95% confidence interval were calculated (only one parameter was estimated for the noninfected group because these data were modelled using an exponential distribution in the simulation in Step 2).

Spearman's rank correlation coefficients of repeated BTM measurements from the same herd were estimated for monthly and 3-month intervals also using data from the Kongeå project. This was also done for infected and noninfected herds as defined above except that for the two herds that changed from infected to noninfected status, data were excluded if collected after the change. Monthly BTM antibody measurements collected from 1 month before the first visit to 1 month after the last visit for each herd were included in the analysis. Within herds, pairs of observations 1, 3, 6, and 9 months apart were identified (measurements occurring within ±15 days of the target interval were included) and then used to calculate Spearman's rank correlation coefficients for each interval and infection status.

Bulk tank antibody and test result simulation model (Step 2)

A simulation model was designed using @Risk software to estimate the probability of testing positive conditional on a 1-year disease history. The inputs for this model were the parameters for distributions of BTM ELISA ODC% for infected and noninfected herds, the rank correlation coefficients for repeated measurements within the same herd estimated in Step 1, and the surveillance program test criteria.

The surveillance program test primarily is based on the results of four sequential BTM ELISA measurements from samples collected at 3-month intervals. However, an automatic retest is performed if a herd previously classified as negative (Level 1) changes to positive (Level 2a) based on the regular sampling scheme. For the retest, a new milk sample is collected about 1 month later and replaces the most recent quarterly value in the test calculations. The use of repeated measurements over time and the nature of the antibody response made it reasonable to assume that surveillance program test accuracy was dependent on the time of sample collection relative to the onset of infection or recovery from infection in the herd. Therefore, the probability of testing positive was calculated separately for herds representing specified infection histories (infected or not infected for each of four consecutive quarters). A quarter of a year was selected as the time unit of interest because (1) tests are usually performed every 3 months and (2) herd infection was assumed usually to have a relatively long duration of months to years. A sequence of infection status representing four quarters of a year was chosen because the herd classification is normally based on measurements from four consecutive quarters. With 2 states (infected and noninfected) and 4 time periods, there were 16 possible sequences of infection and noninfection. These were designated as PPPP, NPPP, NNPP, etc. where P=infected and N=noninfected.

The simulation was used to randomly select a series of 5 ELISA measurements; 4 at 3-month intervals and a fifth value at a 1-month interval (for a potential retest). Values were selected from the distributions corresponding to the infection status for each time period. The fifth measurement was assumed to occur in the same quarter as the fourth. As examples, for herds designated PPPP, five values were randomly selected from the infected-herd distribution and for herds that were noninfected in the first quarter and then infected in the last three (NPPP), one value was selected from the noninfected-herd distribution and then four from the infected-herd distribution. Truncated normal and truncated exponential distributions were used for infected and noninfected quarters, respectively, with distribution parameters based on the field study data analysis described in the previous section. The @Risk correlation feature was used to enter a correlation matrix for each infection sequence with selection of rank correlation coefficients guided by the field study data analysis. Observations from pairs consisting of an infected and a noninfected period were assumed to have zero correlation. Likewise, the correlation was assumed to be zero between pairs consisting of either both infected or both noninfected periods, but separated by at least one period with the opposite status.

The series of five ELISA ODC% values were used to calculate regular test and retest results. The regular test result was coded as 1 if either the average of the 4 quarterly measurements was ≥25 or if the fourth measurement minus the average of the previous 3 was >20 and was coded 0 otherwise. If the regular test was positive, then the retest result was calculated in the same way except the fourth quarter value was replaced with the fifth measurement. The model was run for 10,000 iterations and the proportion positive tests (regular and retest) were calculated for each disease history group. Latin hypercube sampling was used as this method ensures that sampling is from throughout the range of the specified distribution (Vose, 2000).

Test accuracy and predictive value simulation (Step 3)

In Step 3, a separate simulation model was used to estimate the surveillance program test sensitivity, specificity and predictive values for selected herd-level true prevalence values. The scenario pathway diagram for this model is shown in Figure 1. The distribution of herds among infection history patterns was estimated based on a two-state transition model. Movement from the susceptible to infected compartment occurred with infection rate *i* and movement from the infected to the susceptible state with recovery rate r. The recovery rate *r* was equal to 1/(average duration of infection). For the simulation model we assumed constant infection rate, recovery rate, and prevalence over a 1-year period with prevalence equal to $i/(i + r)$ (Alho, 1992). It was also assumed that transitions between states were dependent only on the current state and not on infection status in previous time periods and that loss of herds from the population was independent of infection status.

The minimum, most likely and maximum average duration of herd infection were selected based on clinical experience with infected herds and personal communication with *S*. Dublin researchers. These values (1, 3 and 5 years) were used as parameters for a PERT distribution in @Risk to calculate recovery and infection rates for selected herd-level prevalence. The same duration of infection distribution was used at each value of prevalence. The infection and recovery transition probabilities for a 3-month period were calculated from the corresponding rates as described by Nagelkerke et al. (1990). The probability of each of 16 infection patterns occurring was then calculated as the proba-bility of starting as infected or noninfected multiplied by the transition

probabilities for subsequent states. The model performed the calculations using randomly selected average duration of herd infection (from the PERT distribution) and a specified herd-level prevalence.

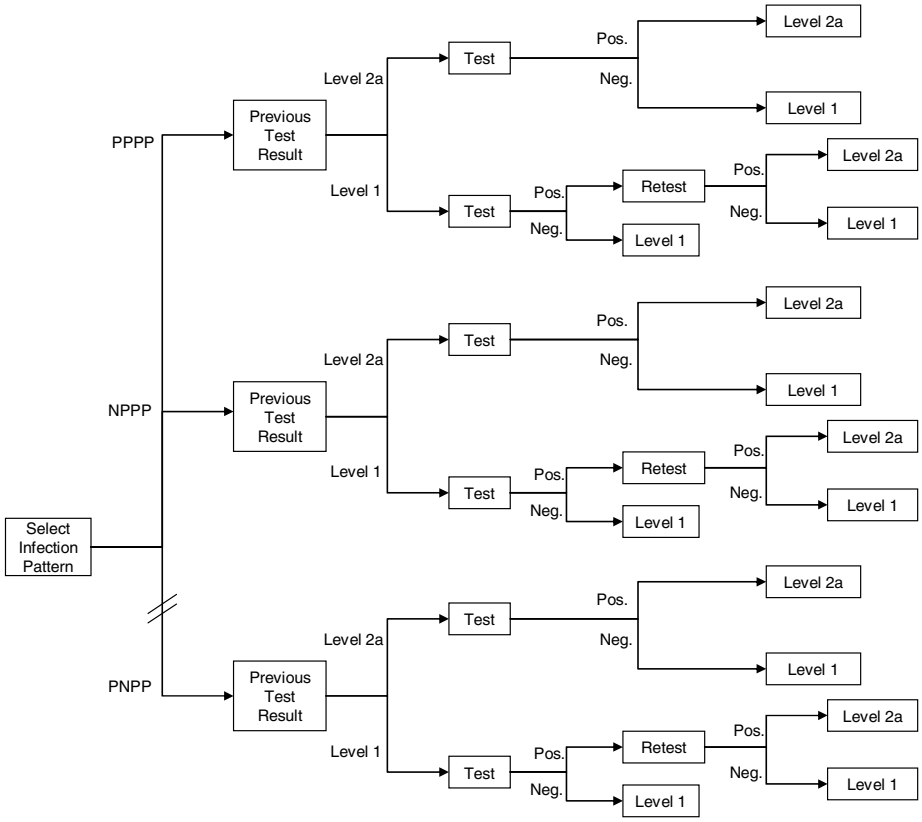

Figure 1 Scenario diagram for a simulation model of test accuracy and predictive values for detecting infected herds in the Danish dairy cattle *Salmonella* surveillance program. Three out of 16 infection history patterns are shown (P = infected and N = noninfected in a series of four quarters of a year). Herds which tested positive were classified as Level 2a and herds which tested negative were classified as Level 1. ''Test'' denoted application of the regularly scheduled program test and ''Retest'' was an automatic retest when a herd previously classified as Level 1 had a positive test result.

Overall program test accuracy and predictive values were calculated with the true positive condition being Salmonella infection in the herd in the current quarter of the year and the true negative condition being freedom from infection in the current quarter. Figure 1 illustrates that there were 5 test result pathways for each of 16 disease history pattern resulting in 80 total pathways. @Risk was used to calculate the probabilities for each pathway using the estimated test result probabilities generated by the simulation described in Step 2. The proportions of herds previously classified 2a was estimated for each of the 16 infection history sequences using an iterative process within each overall model iteration.

This was done by starting with estimates of 0.5, calculating the apparent prevalence for each group, replacing the original estimates with the calculated values, and then repeating the process. The estimates stabilized within 5 iterations with differences between input and output values at that step of <0.0001. The probability of being infected and testing positive and the overall probability of testing positive were calculated as the sum of the probabilities for the Level 2a branches for currently infected and all infection patterns, respectively. The analogous calculations were done using the Level 1 branches for the probability of being noninfected and testing negative and for the overall probability of testing negative. The surveillance program test Se, Sp and positive and negative predictive values were then calculated according to the usual definitions. The test accuracy and predictive value model was run at 5 prevalence values (0.02, 0.08, 0.15, 0.3, and 0.5) with 5000 iterations for each prevalence. Latin hypercube sampling was used for the simulations.

Two important assumptions for the simulation models should be kept in mind. First, for the simulation in Step 2 above, it was assumed that the test results depended on not just the current infection status, but also on the infection status during the past year. This was done because the surveillance program classifies herds based on the results of 4 sequential measurements taken at 3-month intervals. Secondly, for the calculation of the proportion of herds with each of 16 quarterly infection history patterns in Step 3, a first-order Markov process was assumed for the transitions (infection and recovery) between states. Therefore, in contrast to test results that depended on infection history over four quarters of a year (Step 2), the transition probabilities were assumed to depend only on the current infection state (Step 3).

Sensitivity analyses

The potential effects of uncertainty about the distribution parameters and serial correlation coefficients were evaluated through a series of sensitivity analyses where models were run with various combinations of input values. To analyze the effects of input values for the mean and S.D. of the infected herd BTM antibody distribution and mean for the noninfected herd antibody distribution, models were run at selected combinations of the baseline, high and low values. The baseline values were the means estimated from field data and the high and low values were the 95% confidence interval upper and lower bounds for each of the parameters. Models were also run with maxima for the truncated distributions set equal to the analogous maximum observed values from the Kongeå project data or to a value of 250. To evaluate the effect of the rank correlation coefficients for antibody measurements within the same herd, models were run with all correlation coefficients set to 0 or all set to 0.99. Herd-level prevalence was kept at 0.15 for the sensitivity analyses.

Results

Surveillance program results

The surveillance data used for this study consisted of observations from 9378 dairy farms, 7693 of which were still in business in 2003. 23% of 70,914 herd-quarters with at least three preceding BTM ELISA measurements met the criteria for positive test classification (Level 2a). Data collected since the start of the surveillance program showed there was a gradual decline in the percent of dairy herds classified as Level 2a (Figure 2). However, changes from 1 year to the next were only about 1–2 percentage points. The apparent prevalence varied widely by region of the country (Figure 3). For comparison with Kongeå project herds described in the next section, 50 BTM

measurements recorded within 3 months of identification of *S*. Dublin by bacteriological culture in a separate group of surveillance program herds had a mean of 61.8 and S.D. of 25.9 ELISA ODC%. Surveillance program test results were also used to evaluate the probability of herds changing classification levels from one quarter of the year to the next. Among 47,786 Level 1 herd-quarters for which results were available in the following quarter, 2.7% changed to Level 2a. The transition to Level 1 occurred in 11.9% of 14,434 Level 2a herd-quarters. When calculated for individual regions, the Level 1–2a transition probabilities were positively correlated with apparent prevalence (r = 0.97) and had a four-fold increase over a range of apparent prevalence from 7.8 to 39%. The Level 2a–1 transition probabilities were negatively correlated with apparent prevalence (–0.93) and had a two-fold difference between the lowest and highest probabilities.

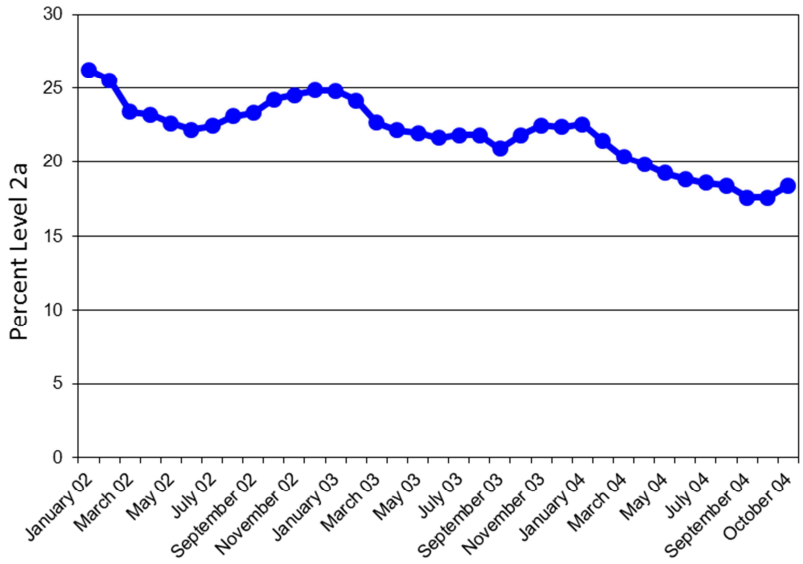

Figure 2 *Salmonella* apparent prevalence in Danish dairy herds over time since the beginning of the Danish National Surveillance program for *Salmonella* Dublin in cattle.

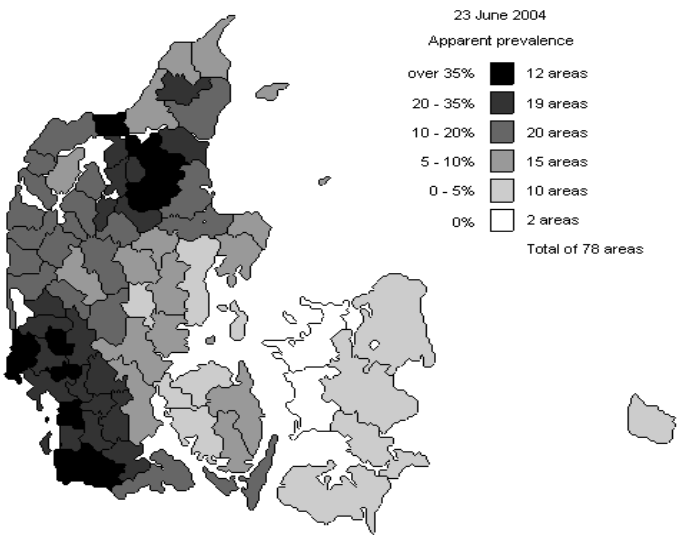

Figure 3 Apparent prevalence of *Salmonella* in June 2004 in dairy herds in Denmark according to bulk tank milk ELISA response measured every 3 months by the Danish National Surveillance Program for *Salmonella* Dublin.

Parameter estimates from the field study

Of 30 field study herds, 7 were classified as noninfected at each visit, 2 initially met the criterion for infection and then changed to noninfected, and 21 were always infected. No herds met the study definition for infection based on fecal culture alone and few herds had within-herd prevalence ≥5% without also having positive fecal samples. Of the 21 herds defined as infected at each visit, 17 had at least 1 positive fecal culture from individual cattle samples, 1 had at least 1 positive environmental sample, but no positive fecal culture, and 3 had no positive cultures from either sample type. Three herds had *Salmonella* Typhimurium infection and no detection of *Salmonella* Dublin by bacteriological culture during the study period. There were no positive individual cattle fecal or environmental samples for herds with <5% within-herd prevalence at any time during the field study period (including the 2 herds that changed status). The distributions of BTM ELISA measurements for infected and noninfected herd visits are shown in Figure 4. The BTM ELISA antibody measurements from infected herd visits (n = 104) were approximately normally distributed (Shapiro–Wilk normality test P-value = 0.6) with mean 65.3 and S.D. of 25.5 while the measurements from noninfected herd visits (n = 31) had a right-skewed distribution with a mean of 7.3 and S.D. of 7.9 (Table 1). The ODC% maxima for infected and noninfected herd visits were 131 and 28, respectively. The distribution parameters as estimated by random resampling with one observation per herd are also shown in Table 1.

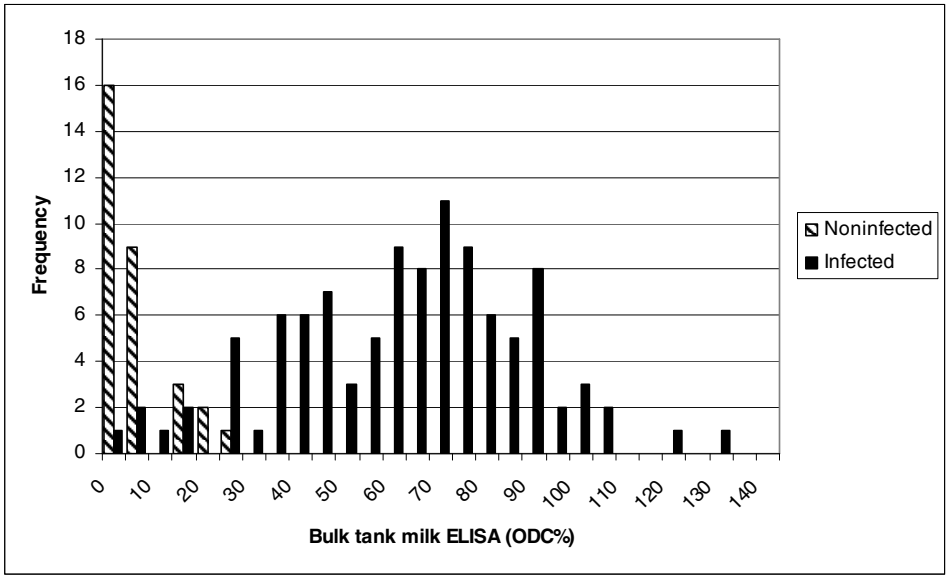

Figure 4 Distribution of bulk tank milk ELISA measurements in 30 dairy herds either infected (positive fecal cultures or ≥5% within-herd apparent prevalence) or noninfected (no positive culture and <5% within-herd apparent prevalence). Data shown include multiple observations per herd.

Table 1 Descriptive statistics for distributions for bulk tank milk *Salmonella* antibody measurements (ELISA ODC%) from 30 Danish dairy herds defined as *Salmonella* infected or noninfected based on individual animal testing at herd visits 3–4 months apart

Raw data	Infected	Noninfected
n	104	31
Mean	65.3	7.3
SD	25.5	7.9
Minimum	0	0
Maximum	131	28
Resampling (100 samples; 1 obs./herd-visit)		
n (herd-visits/sample[a])	23	9
Mean	65.3	8.7
95% CI of mean (LB)	52.8	2.0
95% CI of mean (UB)	77.9	15.4
SD[b]	29.1	-
95% CI of SD (LB)	22.0	-
95% CI of SD (UB)	40.3	-

[a]Two herds contributed data to both the infected and noninfected periods.

[b]Not estimated for noninfected herd measurements which required only 1 parameter for the exponential distribution in the simulation model.

The within-herd rank correlation coefficients for repeated measurements within the same herd tended to be higher for infected herds than for noninfected herds and for the most part were lower for longer intervals between measurements (Table 2).

Table 2 Spearman rank correlation coefficients (rho) for repeated bulk tank milk *Salmonella* antibody measurements from the same herd differing in time by 1, 3, 6, and 9 months in 30 Danish dairy herds defined as infected or noninfected based on individual-animal test results

	Infected		Noninfected	
Lag (months)	*n*	rho	*n*	rho
1	229	0.68	68	0.42
3	130	0.63	42	0.30
6	122	0.53	35	0.32
9	83	0.39	23	0.28

Positive test probability

The input values used for the baseline simulation model in Step 2 were selected based on field data analysis results described above and are shown in Table 3. Correlation coefficients for the noninfected time periods were adjusted so that correlation decreased for longer intervals between measurements. The output from this simulation consisted of estimates for the probability of testing positive for the initial test and retest for each of the 16 four-quarter infection history patterns (Table 4). The probability of testing positive on the initial test was 0.88 or higher for all patterns where the herd was infected in the current quarter. Patterns for herds currently noninfected, but infected in previous quarters had probabilities of testing positive ranging from 0.39 to 0.92 and which increased with higher numbers and proximity of previously infected quarters of the year. For herds noninfected for 4 quarters of a year in row, the probability of testing positive on the initial test was 0.04. Results shown in Table 3 accounted for the variability associated with the distributions of BTM ELISA measurements in infected and noninfected herds and the correlation of measurements within herds, but not for the uncertainty about the distribution parameters. Results of the sensitivity analyses to evaluate distribution parameter uncertainty for this part of the model are shown in Section 3.5 (Sensitivity analyses).

Table 3 Baseline model input values for simulating repeated bulk tank milk *Salmonella* antibody measurements in infected and noninfected Danish dairy herds

	Infected	Noninfected
Distribution	Truncated normal	Truncated exponential
Mean	65.3	8.7
SD	29.1	-
Minimum	0	0
Maximum	200	50
Correlation coefficients		
1 month	0.7	0.40
3 month	0.6	0.35
6 month	0.5	0.30
9 month	0.4	0.25

Test accuracy and predictive values

The output from the test accuracy and predictive model simulation consisted of distributions for Se, Sp, PPV, and NPV for each of five prevalence values. The distribution of PPV at 15% herd-level prevalence is shown in Figure 5 as an example of the output distributions which tended to be left-skewed. The minima, means, and maxima for all test accuracy and predictive value estimates were obtained from the 20 output distributions from the simulation model and are shown in Tables 5 and 6. The ranges for these estimates reflected the effect of uncertainty about herd-level duration of infection.

The mean estimates of Se for the overall test program for detection of herd infection in the current quarter were all equal to 0.95 and had narrow distributions within each prevalence value (Table 5). In contrast, the mean estimates for Sp ranged from 0.83 to 0.98 and were inversely related to herd-level prevalence (Table 5). This occurred because higher prevalence was associated with a higher frequency of irregular infection history patterns (e.g. NPNP, PNPN, etc.) resulting in more false positive diagnoses. The herd-level negative predictive value mean estimates were from 0.94 to 1.0 and the positive predictive value mean estimates were 0.53–0.85 (Table 6). The predictive value results reflected the relatively wide range of Sp and narrow range of Se as well as the effect of prevalence on predictive values.

Table 4 Probability of testing positive in the current quarter for the initial test (P(T1+) and the automatic retest P(T2+|T1+) for 16 infection history patterns at 3-month intervals over 1 year.

Infection Pattern[a] quarter 1, 2, 3, 4	P(T1+)	P(T2+|T1+)
PPPP	0.98	0.99
NPPP	0.95	0.98
NNPP	0.90	0.94
NNNP	0.91	0.95
NNNN	0.04	0.29
PNNN	0.39	0.90
PPNN	0.83	0.98
PPPN	0.93	0.99
NPNP	0.94	0.97
PNPN	0.89	0.98
PNNP	0.94	0.97
NPPN	0.83	0.98
NNPN	0.39	0.89
NPNN	0.39	0.90
PPNP	0.98	0.99
PNPP	0.98	0.99

[a]P=infected; N=noninfected, quarter 4=most recent quarter of the year.

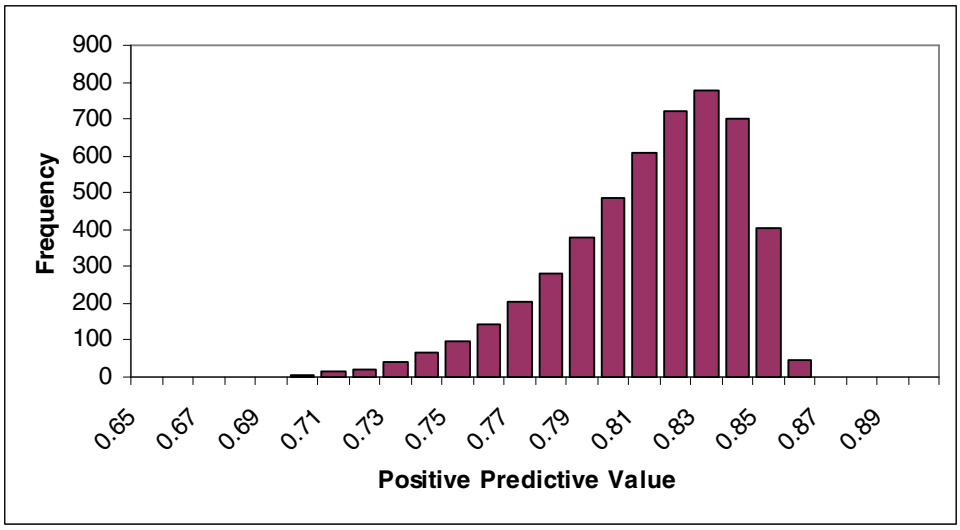

Figure 5 Frequency distribution for the positive predictive value of a bulk tank milk antibody ELISA for the diagnosis of *Salmonella* infections in Danish dairy herds as an example of simulation model output distributions. The data are from 5000 iterations at a herd-level infection prevalence of 15%.

Table 5 Estimates of sensitivity (Se) and specificity (Sp) for determining *Salmonella* infection status in dairy herds at five different herd-level prevalence values in the Danish *Salmonella* surveillance program

	Se			Sp		
Prevalence	Min	Mean	Max	Min	Mean	Max
0.02	0.93	0.96	0.96	0.98	0.99	0.99
0.08	0.94	0.96	0.96	0.96	0.97	0.98
0.15	0.94	0.96	0.96	0.92	0.96	0.97
0.30	0.94	0.96	0.96	0.84	0.92	0.95
0.50	0.95	0.96	0.97	0.68	0.83	0.89

Sensitivity analyses

Sensitivity analyses were run to evaluate the effect of uncertainty about model input parameters on test accuracy and predictive values at a herd-level prevalence 0.15. Se mean estimates ranged from 0.85 to 0.996, Sp from 0.85 to 0.98, PPV from 0.52 to 0.89 and HNPV from 0.97 to 0.999 over various combinations of input parameters. The most important changes in predictive value estimates occurred for PPV. The PPV decreased substantially if either the serial correlation of antibody measurements within herds was assumed to be high or if the mean of the antibody distribution for the noninfected periods was higher than baseline. PPV increased with low serial correlation or low noninfected herd mean antibodies. High 1-month correlation in negative herds decreased Sp and PPV by causing an increase in the proportion of positive retests in herds noninfected for 4 quarters in a row.

Table 6 Estimates of positive predictive value (PPV) and negative predictive value (NPV) for predicting *Salmonella* infection status in dairy herds at five different herd-level prevalence values in the Danish *Salmonella* surveillance program

Prevalence	PPV			NPV		
	Min	Mean	Max	Min	Mean	Max
0.02	0.50	0.57	0.60	1.00	1.00	1.00
0.08	0.65	0.76	0.81	0.99	1.00	1.00
0.15	0.68	0.81	0.86	0.99	0.99	0.99
0.30	0.72	0.84	0.89	0.97	0.98	0.98
0.50	0.75	0.85	0.90	0.93	0.95	0.96

Discussion

Previous research and development of surveillance program

The testing program we evaluated is part of a surveillance program developed to contribute to the control *S*. Dublin infections in Danish cattle herds. One feature of the program is that herd classification is made publicly available so that the status of a herd can be considered in cattle purchasing decisions. Important effects of misclassification in the surveillance program may include purchase of infected animals from herds incorrectly classified as Level 1 and unnecessarily, at least with respect to Salmonella, limiting purchase of cattle from noninfected herds falsely classified as Level 2a. This is the first study to estimate the frequency of misclassification in the Danish *Salmonella* surveillance program for dairy herds.

Previous field and laboratory studies addressed herd-level *S*. Dublin testing, but are not directly comparable to our results (Hoorfar and Bitsch, 1995; Hoorfar et al., 1994; Veling et al., 2002; Wedderkopp et al., 2001). None of these studies evaluated test classification of herds based on BTM ELISA from repeated samples from the same herds. Instead comparisons were made between a single BTM ELISA measurement for each herd and herd infection status defined either by bacterial culture results or on serology of individual animals. A *S*. Dublin control program evaluation project was carried out by Danish researchers before instituting the national surveillance program (Anon, 2001). The study design did not provide valid estimates of test accuracy and predictive values of the test as implemented in the current program or allow inference to the general Danish dairy herd population. Nevertheless the evaluation project results and other experiences with the BTM ELISA provided information to guide the selection of test criteria. The priority for the surveillance program was to achieve high Se and thereby high NPV so that at least the negative test results could be considered reliable and provide a tool for farmers to avoid purchasing cattle from infected herds. In addition to the effect of cut-off values chosen, allowing positive tests for either a high 4-quarter moving average or a large increase between the most recent test and the previous 3-quarter moving average allowed detection of gradual as well as sudden increases in antibody concentrations and had the effect of increasing Se and NPV. Depending on the amount of correlation between measurements from the same herd, using test criteria based on the average of repeated measurements has the potential advantage of reducing the variability of the test parameter and thereby increasing test accuracy. This benefit must be weighed against the disadvantage of errors which can result from the influence of past infection status and previous test results on current classification. This type of testing program based on moving averages is most appropriate for herd infections of relatively long duration.

Assuming a true herd-level infection prevalence of 15%, under the current Danish surveillance system about 16–32% of Level 2a dairy herds would be noninfected and less than 1% of Level 1 herds would actually be infected with *Salmonella* as defined for the simulation study. The general agreement between bacteriological culture results and antibody measurements in the field study herds suggests that the disease definition used for the simulation is relevant to detecting herds with *Salmonella* infected cattle that are shedding bacteria in the feces at least intermittently. The effects of herd *Salmonella* culture results, within-herd prevalence and other factors on BTM antibody measurements were reported previously for a project using most of the same herds as were used for our study (Nielsen and Ersbøll, 2005). The amount of misclassification estimated in the simulation study was consistent with the goal of decreasing the risk of transmission from cattle purchased from Level 1 herds. However, from a cattle seller's point of view, it may represent a relatively high probability of being free of infection when classified as Level 2a.

Based on results of this project the surveillance test program has since been modified to allow individual animal testing in Level 2a herds in order to decrease the amount of false positive classification. While NPV estimates were generally very high, they decreased with high herd-level prevalence suggesting that purchase from Level 1 herds in high-prevalence regions could still represent an important risk of buying infected cattle.

Evaluation of model input assumptions

The results of the simulation model should be interpreted in light of the model design and assumptions about input values. The uncertainty about model parameters was either modeled by a random distribution (average duration of herd-level infection) or evaluated by performing sensitivity analyses. The relatively small number of herds used to estimate the distribution of BTM measurements, especially for the noninfected group, could have resulted in inaccurate input distribution parameters. For example, the selection of the maximum values to truncate the distributions of BTM measurements was somewhat arbitrary. Unbounded distributions were not used because out of approximately 100,000 measurements in the surveillance program data, the maximum BTM ELISA measurement observed was 204. Sensitivity analysis of the effect of distribution maxima were run using either the maximum observed values from the Kongeå project or a value of 250 ODC%. These models showed almost no change in Se and NPV. This would be expected because the moving 4-quarter average test cut-off was to the left of the center of the infected herd distribution so extension of the right tail would not have a large effect on the probability of exceeding the cut-off. The effect was slightly larger on Sp and PPV but the range was still relatively narrow with Sp estimates of 0.95 and 0.97 and for PPVof 0.77 and 0.85 for the alternative assumptions.

The similarity between BTM ELISA distributions from the Kongeå project infected herds and from a separate sample of culture-positive herds from the surveillance program increased confidence in the infected herd distribution parameters used for the model. There was less certainty about the noninfected distribution because no data were available other than from the herds tested in the Kongeå study. The effect of BTM antibody distributions on model results was evaluated using various combinations of the minimum, maximum and mean values for distribution parameters. The sensitivity analyses showed that NPV estimates were not substantially different over the range of input parameters tested. PPV was more sensitive to these parameters and had a wider range with the lowest estimate for the mean being 0.52. The lowest estimate occurred when the

infected herd mean was at a very low value and the infected herd S.D. and noninfected herd beta were at very high values. This would of course result in a much greater overlap of the distributions than was observed in the field study.

The selection of correlation coefficients also had a greater effect on PPV then NPV. For example, mean PPV was 0.65 and 0.83 for assumptions of 0.99 and 0.0 correlation coefficients, respectively. For the same scenarios, NPV was 0.98 and 1.0. Small errors in the specification of the correlation coefficients were unlikely to seriously affect the model results, particularly because the true correlation coefficients were probably not negative or near 0 or 1.

Effects of model design

Several other important assumptions were related to the model design rather than the values of the input variables. The first simulation module was based on the assumption that the same distribution of BTM measurements applies to all infected herd-quarters, regardless of the time since infection. The analogous assumption was made for the exponential distribution for the noninfected herd-quarters. In other words, we modelled an abrupt rather than gradual change in distributions when infection status changed. The model was designed in this way because the field data were not adequate to estimate the positive and negative BTM distributions separately for various times since infection or times since recovery from infection. If, in fact, the true distributions depend on time since infection we would expect the model would have overestimated Se (because the mean BTM measurement was specified to be higher than the true distribution) and underestimated Sp (because the mean BTM measurement for quarters just preceding recovery was specified to be higher than the actual distribution). These biases would affect irregular infection history patterns and would only have a substantial impact on the overall test accuracy and predictive value estimates under high prevalence and incidence conditions where the proportion of irregular patterns is increased.

Another feature of the model was that zero correlation was assumed for pairs consisting of one noninfected quarter and one infected quarter. The same applied to pairs of infected quarters separated by at least one noninfected quarter and for pairs of noninfected quarters separated by at least one infected quarter. The reasoning behind this assumption was that the conditions contributing to serial correlation (e.g. sampling a herd consisting mostly of the same group of noninfected cows) would have a small impact on measurements relative to the effect of changing infection status. Also, true changes in herd-level states of infection (e.g. recovery followed by re-infection and subsequent recovery) would imply that a large scale change occurred in the infection status of individual animals or that large numbers of animals were sold or purchased. Our assumption was that the effect of taking repeated measurements in the same herd would be small in comparison to effects of herd-level changes in infection status.

The simulation model used prevalence, incidence and recovery rates to calculate the expected frequencies of 16 disease history patterns. The model inputs were duration of infection (modeled as a PERT distribution) and fixed values for prevalence. The incidence was calculated from prevalence and recovery rate. For this method, the average duration of infection was assumed to be independent of prevalence. To evaluate this assumption, we estimated transition probabilities (Level 1–2a and Level 2a–1) as proxies for incidence and recovery rates. This was done separately for seven regions with a wide range of herd level apparent prevalence values. The probability of the 1–2a transition increased approximately 4-fold over apparent prevalence values ranging from

7.8 to 39%. This was consistent with the model assumption that increased prevalence was attributable to increased incidence. Over the same range, the probability of the 2a–1 transition decreased by about a factor of 2 which corresponds to a change in estimates for the average duration of Level 2a status from about 1–2.5 years. The relationship between apparent prevalence and changing from Level 2a status may be explained in part by misclassification. For example, the average Level 2a duration could be shorter in low prevalence areas where a greater proportion of 2a herds are actually free of infection. Although an effect of a true relationship between the duration of infection status and herd-level prevalence cannot be ruled out, modeling the average infection duration with a random distribution ranging from 1 to 5 should have been sufficient to account for this in the simulation model.

The method for calculation of infection history frequencies also depended on assumptions of constant prevalence, independence of infection status and exit from the population, and independence of transition probabilities from prior disease history. Changes in apparent prevalence over the course of the surveillance program showed there may have been a seasonal pattern with higher apparent prevalence in the late Fall and a gradual decline over several years. Assuming test performance was constant, this would imply a decline in true prevalence as well. However, the change in prevalence over 1 year was probably not large enough to cause important changes in the misclassification mode results or conclusions. The assumptions of independence of herd exit from infection status and the independence of transition probabilities from past infection history could not be evaluated from observational data as part of this project, but were considered to be reasonable simplifying assumptions for the modeling process.

Conclusion

Field data and simulation were used to estimate the test accuracy and predictive values for the testing program used in the Danish *Salmonella* surveillance program for dairy cattle. Herd classification was based on *Salmonella* antibody concentrations in a series of four BTM samples collected at 3-month intervals. The surveillance program and simulation study results illustrate the challenge in balancing positive and negative effects of using summary measures of repeated test results for herd infection classification. Under the model assumptions and using test criteria applicable to the time of field data collection, accuracy and predictive value estimates were dependent on herd-level *Salmonella* prevalence. At herd-level prevalence of 15%, Se, Sp, PPV, and NPV were 0.95, 0.96, 0.80, and 0.99, respectively. Although the PPV was somewhat low, this amount of misclassification was consistent with the surveillance program goal of having high confidence in a negative test result.

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PAPER XV

A structured approach to control of *Salmonella* Dublin in 10 Danish dairy herds based on risk scoring and test-and-manage procedures

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A structured approach to control of *Salmonella* Dublin in 10 Danish dairy herds based on risk scoring and test-and-manage procedures

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Abstract

The aim of this study was to describe a structured approach to effectively reduce *Salmonella* Dublin prevalence in infected dairy herds based on a step-wise procedure. Furthermore, the aim was to describe tools for management and monitoring, and to report on development in prevalence among young stock and adult cattle in 10 case herds that were followed for more than three years.

The five steps in the structured approach were: 1) risk scoring to determine transmission routes within the herd and into the herd; 2) determining a plan of action; 3) performing management changes to close important routes of infection; 4) interpretation of repeated testing of individual animals to detect high-risk animals for special hygienic management or culling; and 5) diagnostic testing of different age groups and bulk tank milk to evaluate progress of control over time.

Serology, true prevalence estimates and changes in herd classification in the Danish surveillance programme for *Salmonella* Dublin were used to assess the progress in the herds during and after the control period. Effective control of *Salmonella* Dublin was achieved in all participating herds through management that focused on closing infection routes mainly in the calving areas and the young calf areas of the herds. It took on average three years to control the infection in the case herds. Bulk tank milk recordings from the four following years indicated that most of the herds might have eradicated the infection.

Introduction

Salmonella enterica subsp. *enterica* serovar Dublin (*Salmonella* Dublin) is a bacterium that receives much attention in the cattle industry of several countries around the world due to a relatively high prevalence in cattle dense areas and potentially severe zoonotic implications for humans and animal health, welfare and production (Peters, 1985; Helms et al., 2003; Nielsen et al., 2010). Therefore a national surveillance programme was initiated in Denmark in 2002. In this programme, all cattle herds are tested on a regular basis and placed into one of the three classification levels (Warnick et al., 2006). Furthermore, farmers in infected herds are encouraged through trade restrictions to control the infection. Herd-specific approaches are required to control the infection, which is often latent in a number of animals that shed the bacteria intermittently. Previous attempts to control *Salmonella* Dublin in dairy herds have proven difficult for farmers.

Approximately half of the dairy herds that experience an outbreak of *Salmonella* Dublin become persistently infected (Veling, 2004a). A number of infected animals have a latent infection with intermittent bacterial shedding in faeces (Richardson, 1973; Wray et al., 1989) and some animals might become active carriers, i.e. animals that shed bacteria more or less continuously for extended periods of time (House et al., 1993). There are indications in field studies that persistent infections at herd level cannot be stopped solely by culling active carriers detected by bacteriological isolation of *Salmonella*-bacteria in faecal samples (Veling, 2004a). Furthermore, test-and-cull strategies might not be cost-effective, especially if within-herd

transmission can be prevented without the culling of potential carriers. Vaccination is used as a tool to control *Salmonella* Dublin in several countries. However, vaccination is mainly useful for reducing clinical signs and shedding of bacteria from affected animals. It does not entirely stop bacteria from spreading to the environment and between animals (Segall and Lindberg, 1993; Mizuno et al., 2008).

Calves below the age of 3 months are more susceptible to *Salmonella* Dublin than older animals leading to more severe clinical signs and more shedding of bacteria in this age group (Nazer and Osborne, 1977; Segall and Lindberg, 1991). Usually, the incidence rate of acute infections is highest in this age-group, but infection (including persistent infection) can occur at any age if the infection dose is sufficiently high. Therefore, control of *Salmonella* Dublin needs to be focused on stopping transmission to and between young calves, but also to lower the infection pressure in general in the herd environment. Furthermore, an important part of a control programme is to avoid purchase of infected animals from other herds (Nielsen et al., 2007b). In the Danish surveillance programme for *Salmonella* in cattle this is done by providing farmers with access to information about the status of all other cattle herds via the internet.

In a previous study, six persistently infected dairy herds started a voluntary control effort based on changes in management at calving and of the pre-weaned calves. In addition, all adult cattle (>1 year) were monitored serologically and cows with persistently high *Salmonella* Dublin antibody titres were recommended culled (Jensen et al., 2004). Five of the herds managed to control the infection and reduce the proportion of seropositive animals markedly. One herd had a recurrence of salmonellosis four years after the control effort was initiated. The study showed that several of the herds had difficulties carrying through the recommended management changes, and they also purchased new animals from herds with unknown infection status during and after the study period (Jensen et al., 2004). Thus, there appeared to a need for tools to assist farmers wanting to control *Salmonella* Dublin in their herds.

The aim of this study was to describe a structured approach to effectively control *Salmonella* Dublin in infected dairy herds based on a step-wise procedure provided in a manual for farmers and advisors. The approach incorporated didactic principles to assure the necessary commitment and follow-up during the control period required to obtain daily and long-term control efforts. Furthermore, the aim was to describe tools for management and monitoring, and to report on development in seroprevalence among young stock and adult cattle in 10 case herds. Monitoring was done by repeated serological testing of all animals in the participating herds during a three-year period starting in 2003, and through observation of herd classifications in the Danish surveillance programme between 2001 and 2010. The study was designed for illustrative purposes. Thus, it is a descriptive case-study that does not allow for comparison of the effect of different management strategies between the herds.

Materials and methods

Herds

The study was initiated in June 2003 and the participating herds were followed intensively until the end of 2006. After that the herds were monitored via the Danish national surveillance programme for *Salmonella* Dublin in cattle. A convenience sample of 10 dairy herds was found through veterinary practitioners. The herds were located in several regions of the main Danish peninsula, Jutland. The herds were initially all placed in surveillance Level 2 indicating too high antibody levels in bulk tank milk ELISA measurements, and thus likely infected with *Salmonella* Dublin. The surveillance programme classifications are described in

more detail in section 2.6 and have also been described and evaluated elsewhere (Warnick et al., 2006). Table 1 gives an overview of the participating herds.

Didactic risk scoring tool

A tool was developed to assist farmers and cattle health consultants identify transmission routes in the herds and plan control strategies accordingly. This manual was based on ideas from an American manual concerning paratuberculosis control (Rossiter et al., 1999). It was adjusted to match the infection dynamics of *Salmonella* Dublin and Danish farming practises and legislation. The idea was to use risk scoring to go through the herd systematically from one end to the other and assign risk scores to different relevant barn sections and management practises in the current system on a scale from 0 to a maximum score. The maximum scores were decided by the authors of the manual according to existing knowledge about risk factors for spread of *Salmonella* Dublin in cattle herds, and they were weighted so that the most critical areas for control counted most in the total sum of risk scores (e.g. hygiene and management in the calving area and the pre-weaned calf barn). The scoring system is not very rigid, so the absolute numbers can probably not be used for statistical purposes, but discussions on what each score should be and why stimulate communication between the farmer and his advisor(s). Thus, the manual with both background information and the scoring system was developed as a didactic tool to focus attention of decision makers in the farm on the most critical control points in each specific herd. An English version of the *Salmonella* Dublin risk scoring form is available in the online supplementary material (Risk_scores_Salmonella.xls).

Plan of action

The next step was to summarise and evaluate the risk scores and to make a plan of action based on the results. Visual presentations of the summary risk scores for each area in the herd were implemented in the risk scoring forms (see supplementary material: Risk_scores_Salmonella.xls). The plan should always contain i) a clear statement of the action, e.g. calf pens to be cleaned, disinfected and dried out for 2 days before a new calf is allowed into the pen; ii) the name of the person responsible for the action in the herd; iii) a date for when this action should be implemented, and iv) an agreement about follow-up on the action. It was recommended that the farmer made this plan of action in collaboration with a herd health consultant, and that they planned regular follow-up on the plan, e.g. every 6 months during the control period. It was an important part of the didactic idea behind the manual that the farmer was actively involved in deciding the plan of action, so it should not be dictated by the advisor.

Table 1 Overview of 10 dairy herds that participated in a Danish *Salmonella* Dublin control study in 2003-2006.

Herd	Date herd started in project[a]	Date Level 1 was obtained[b]	Herd size (#cows) at start and end of study period		Main breed	Sero-prevalence in young stock[c] at first and last sampling round		Sero-prevalence in lactating cows[d] at first and last sampling round	
	Month year	Month year	Start	End		Start	End	Start	End
A	Oct. 2003	April 2004	97	170	Danish Holstein	1%	1%	10%	1%
B	Jan. 2004	Feb. 2006	112	157	Danish Holstein	1%	0%	27%	0%
C	Feb. 2004	Oct. 2004	65	74	Danish Holstein	4%	0%	6%	1%
D	Oct. 2003	Mar. 2005	88	91	Danish Holstein	6%	1%	13%	1%
E	Dec. 2003	May 2006	71	102	Danish Jersey	7%	0%	26%	1%
F	Oct. 2003	Sep. 2005	98	120	Danish Holstein	9%	1%	30%	0%
G	Dec. 2003	July 2006	189	201	Danish Holstein	31%	3%	25%	5%
H	Oct. 2003	Feb. 2008	88	116	Danish Holstein	9%	23%	40%	4%
I	Dec. 2003	Nov. 2007	96	136	Danish Holstein	21%	3%	20%	8%
J	Dec. 2003	Oct. 2008	68	67	Danish Holstein	57%	5%	41%	21%

[a] Defined as date of first individual milk sampling.

[b] Level 1 indicates "most likely free from Salmonella Dublin-infection" in the national surveillance programme. Herd A returned to level 2 in January 2009 due to increases in bulk-tank milk antibodies.

[c] Based on screening of antibodies in serum of all animals N3 months in small and medium sized herds, and animals 3–7 months and heifers N15 months in large herds.

[d] Based on screening of antibodies in milk of all lactating cows.

Recording of management changes

The third step was to perform the planned actions. All herds were visited towards the end of the study period in 2006 and a semi-structured interview was performed by the authors of this manuscript. In Table 2 is an overview of the main management practises and changes that were recorded in each herd of relevance for *Salmonella*-control. Note that the sample size of herds does not allow for comparison of management practises between herds, so the listed management practises performed in the herds should mainly be seen as actions that were feasible for the farmers to perform during the control period.

Table 2 Control actions, starting dates and time to success for 10 Danish dairy herds in a *Salmonella* Dublin field study

Herd	Start date of intervention actions[a]	Main intervention actions	Time until effect on young stock[b]	Time until reaching level 1[c]
A	November 2003	Camera surveillance of calvings with fast removal of calves from high-risk cows. From September 2005 calves moved to outdoor calf hutches, heifers moved to heifer raising facilities off premises. Culling of persistently high-titre cows.	0	8
B	January 2004	The first 1½ years of the intervention period calves were removed from the dam immediately after birth. No milk used to feed calves from high-risk cows. Heifers moved to heifer raising facilities off premises.	0	25
C	January 2004	Calves were removed from the dam 1-7 h after birth. Immediate removal of calf if cows appeared on high-risk list.	0	9
D	January 2004	No milk fed to calves from high-risk cows. Thorough cleaning of pre-weaning calf pens before introducing new calves. Hygiene in pre-weaned calf pens and feeding buckets improved.	0	14
E	January 2003	Outdoors calf hutches. Removal of calf from dam 1-7 h after birth. Culling of persistently high-titre cows, but not all persistently high-titre heifers from January 2004.	15	41
F	January 2004	Single calving pens cleaned after every calving. Calf removed immediately after birth. Thorough cleaning of outdoor calf hutches, before new calves were moved in. Culling of high-risk cows when convenient.	22	20
G	November 2004	Pre-weaning calf pens cleaned between each calf. Discontinued use of high-pressure cleaning indoors. All-in-all-out for all group housing calf sections. Strict management of colostrum with bank of milk from low-risk cows fed via a tube. Common calving area split into two – one for high-risk cows and one for others from July 2005. Culling of persistent high-titre animals in late stages of intervention.	24	20
H	October 2003	Calves removed immediately after birth. Pre-weaning calf pens cleaned between each calf. Persistently high-titre cows culled if convenient.	N/A	52
I	July 2004	Heifer calves removed immediately after birth. Only milk replacement fed to calves.	28	41
J	May 2004	Calving moved to outdoors. Fast removal of calves from high-risk cows at calving. Pre-weaned calves moved to outdoor calf hutches, however, not consistently.	30	53

[a] Estimated from interviews with farmer

[b] Number of months from intervention started to young stock seroprevalence was below 5%

[c] Number of months from intervention started to level 1 was obtained. Level 1 indicates "most likely free from *Salmonella* Dublin-infection" in the Danish surveillance programme

Diagnostic tests on animal level

All lactating cows had individual milk samples collected every 3 months. Blood samples were collected twice per year from all heifers from 3 months of age up to first calving for prevalence estimation in the young stock. All of these samples were tested using an indirect ELISA detecting antibodies to *Salmonella* Dublin lipopolysaccharide O-antigens. The ELISA has been described and evaluated in other studies (Nielsen and Ersbøll, 2004; Nielsen et al., 2004). The individual ELISA results were provided to the farmers in two ways: 1) progress graphs to evaluate if the plan of action was working and 2) colour-coded decision support lists indicating high-risk animals for special management (i.e. single calving pen with rigorous cleaning) or culling. The classification of the individual animals was done based on repeated ELISA-measurements and modified from (Smith et al., 1989; Spier et al., 1990; House et al., 1993). Animals were classified risk group R=1 (marked in red on the list), if they had at least two samples above 80 ODC% with a minimum of 120 days in between, *and* the most recent sample was above 80 ODC% , *and* the average of the last up to four samples was above 80 ODC%. The animals were categorised medium risk indicated by R=2 (marked in yellow on the list), if the most recent ELISA and the average of the last up to four samples were above 50 ODC%, but not high enough to be categorised as R=1. Animals with ELISA values below 50 ODC% in the most recent sample did not have any colour indicators on the decision support lists.

Diagnostic tests on herd level

In the national surveillance programme for *Salmonella* Dublin all dairy herds automatically had a bulk tank milk sample collected approximately every 3 months from 2001 and onwards. These were tested for antibodies against *Salmonella* Dublin with a bulk tank milk ELISA described elsewhere (Nielsen and Ersbøll, 2005b). Herds were classified into three levels based on the average ELISA values of the last four samples, and the value of the latest sample in relation to the average of the previous three. Furthermore, movement of animals and detection of bacteria usually upon clinical suspicion could affect herd classification. The programme validity has been estimated by (Warnick et al., 2006). It was found that at a true prevalence of 15%, approximately 1% of Level 1 herds were false negative meaning that they might be infected with *Salmonella* Dublin despite low bulk tank milk antibody levels. It was also found that approximately 20% of the Level 2 herds were in fact not infected with *Salmonella* (or had too low prevalence for it to be detected), but still had too high antibody levels to be assigned to Level 1 .

Statistics

The true prevalence (TP) at each sampling round was estimated from the apparent prevalence (AP, the number of animals with ELISA-values >50 ODC% out of all tested animals), the sensitivity (Se) and specificity (Sp) estimates by formula (1) (Houe, Ersbøll & Toft, 2004):

$$TP = AP + Sp - 1 / Sp + Se -1 \quad (1)$$

The estimates of test validities used in these calculations were Se=0.75 and Sp=0.95 for young stock (serum ELISA) and Se=50% and Sp=90% for adult cows (milk ELISA) (Nielsen, 2003a; Nielsen and Ersbøll, 2004; Nielsen et al., 2004).

Paired T-tests were used to compare the within-cows and within-heifer true prevalence estimates at the first and the last sampling rounds across all herds.

Results and discussion

Control actions performed in the study herds

Herd characteristics and main performed control actions are summarised in Tables 1 and 2. The date that control actions were initiated was estimated from the farmer interviews. However, it was not possible to determine an exact start date, because some control actions were not started simultaneously within the herds. Farmer behaviour varied markedly between the herds. Herd E had already initiated control of *Salmonella* Dublin 10 months prior to the first sampling, but otherwise control actions were generally started after the time of entry into the study. Herd G did not start control actions in the calf barn until one year into the project. Herds I and J did not consistently stick to the planned control actions. In particular, they were not consistent in the use and management of calving areas and single pen housing of calves. Herd H, on the other hand, appeared to be doing very well with regard to control actions and the farmer was very motivated to reach Level 1 as quickly as possible in order to be able to sell high quality breeding animals. He was therefore very consistent in removing calves immediately after calving irrespective of calving hour and in cleaning of calf pens between calves. Fast removal of calves from the dam after birth reduces the risk of calves becoming infected from their own mother or from other cows in the calving area (Richardson, 1973). There was, however, a period with indications of new infections among the young stock and cows during winter 2005 in Herd H. Such reoccurrence of new infections might be due to presence of asymptomatic carrier animals in the herd or to surviving infection in the environment (Taylor and Burrows, 1971; House et al., 1993; Plym-Forshell and Ekesbo, 1996; Veling, 2004a). None of the herds engaged in major new barn construction projects during the project period, but some rearranged the interior of the barns to obtain a better flow of animals or improved possibilities for cleaning and group sectioning.

Evidence for major risk factors of within-herd transmission to support advices for optimal control strategies is often based on empirical knowledge (Richardson, 1973; Hardman et al., 1991; House and Smith, 2004). A major reason for lack of scientific evidence is that comparison of management and housing systems in scientific field studies poses major challenges, not least in longitudinal studies. Field studies of infections that mainly spread through faecal to oral routes require expensive and time-consuming sampling with frequent testing of both animals and environment, and recording of management procedures and changes hereof over long periods of time to lead to significant new knowledge about infection dynamics and within-herd risk factors of *Salmonella* Dublin (Nielsen et al., 2007a). Alternatives to observational studies are simulation modelling of within-herd infection dynamics. However, such models also require reliable input parameters and prior knowledge about risk factors, and often mainly provide hypothetical or theoretical conclusions (Xiao et al., 2005).

Salmonella Dublin can be transmitted from infectious animals to most age-groups suggesting that strict group-housing is recommendable, and the highly susceptible pre-weaned calves should be housed in clean environment. It should be considered that any manure originating from other animals should not come into contact with susceptible animals (Hardman et al., 1991). Alternative isolated pens for - or culling of - sick animals may be preferable during control, because clinically affected animals shed highest numbers of *Salmonella*-bacteria (Segall and Lindberg, 1991).

Diagnostic tests on animal level

The ELISAs used here have been evaluated for sensitivity and specificity with regard to infection in the individual animal (Nielsen, 2003a; Nielsen and Ersbøll, 2004; Nielsen et al., 2004). Estimated sensitivity of approximately 50-88% and specificity of around 88-98% at cut-off 50 ODC% depending on the age of the tested animal suggest that serology has its strength in group or herd diagnostics or if interpreted based on repeated sampling of the same animals.

Bacteriological culture for *Salmonella* has very poor sensitivity (maybe as low as 6-14%) in subclinically infected cattle (House et al., 1993; Nielsen et al., 2004). Consequently, infections in some groups of animals or even in the whole herd would go unnoticed, if bacteriology had been used for evaluation of progress in the control of *Salmonella* Dublin in this study. The sensitivity is poor due to intermittent shedding, low concentrations of bacteria in the samples and other factors related to the origin of the faecal material and the strain of bacteria (Baggesen et al., 2007a). Thus, use of bacteriology would not provide sound conclusions, and it was decided to use the more cost-effective and sensitive ELISAs in this study.

Farmers were provided with three decision support tools based on individual animal diagnostic testing during the study period:
1) sorted lists of diagnostic test results for all animals tested in the herd (Table 3);
2) high-risk animals identified by fixed criteria as described in the Materials and methods section (Table 4);
3) graphs illustrating the antibody ELISA response in the samples versus the age of the animals on the date of sampling (Figure 1).

These tools were sent out to farmers four to six times per year approximately one month after each sampling round. It was not possible to obtain accurate information about which animals were culled or managed according to these lists in this project. However, in a culling analysis of these herds, it was found that the risk of culling was significantly higher across all herds for heifers and cows that had been classified as R=1 or R=2 during the study (Nielsen and Dohoo, 2010). The types of graphs illustrated in Figure 1 based on sampling of large groups of cattle were highly appreciated by the farmers. However, they require sampling of a large number of animals which is time consuming and expensive. Smaller samples of indicator groups such as calves between 4 and 6 months of age have been suggested by others to be useful for herd diagnostics (Veling et al., 2002).

Table 3 Example of list of repeated antibody ELISA results from all tested cattle in *Salmonella* Dublin control herds

Animal ID	Age in years	ELISA 1 (date)[a]	ELISA 2 (date)	ELISA 3 (date)	ELISA 4 (date)	Average[b]
934	3.8	4 (02AUG06)	0 (17MAY06)	0 (15FEB06)	0 (04JAN06)	1
936	3.7	0 (02AUG06)	0 (17MAY06)	0 (15FEB06)	3 (04JAN06)	1
941	7.4	38 (02AUG06)	6 (17MAY06)	21 (15FEB06)	3 (04JAN06)	17
954	3.3	15 (02AUG06)	5 (17MAY06)	18 (15FEB06)	3 (04JAN06)	10
955	3.3	11 (02AUG06)	34 (17MAY06)	10 (15FEB06)	10 (04JAN06)	16
956	3.3	7 (02AUG06)	0 (17MAY06)	0 (15FEB06)	2 (04JAN06)	2
983	5.5	9 (02AUG06)	1 (17MAY06)	.	.	5
984	5.5	25 (02AUG06)	7 (17MAY06)	15 (15FEB06)	6 (04JAN06)	13
990	6.2	16 (02AUG06)	18 (17MAY06)	10 (15FEB06)	21 (04JAN06)	16
991	6.2	17 (02AUG06)	6 (17MAY06)	48 (04JAN06)	17 (01SEP05)	22
993	5.4	3 (02AUG06)	7 (17MAY06)	8 (04JAN06)	1 (01SEP05)	5
999	5.3	17 (02AUG06)	0 (17MAY06)	18 (15FEB06)	14 (04JAN06)	12
1001	5.0	30 (17MAY06)	4 (15FEB06)	0 (04JAN06)	.	11
1012	4.7	0 (02AUG06)	0 (17MAY06)	0 (01SEP05)	0 (21MAR05)	0
1013	4.2	24 (15FEB06)	27 (04JAN06)	2 (01SEP05)	10 (16JUN05)	16
1035	4.7	32 (02AUG06)	20 (15FEB06)	22 (04JAN06)	15 (01SEP05)	22
1038	4.6	27 (02AUG06)	18 (15FEB06)	18 (04JAN06)	10 (01SEP05)	18
1039	4.6	0 (02AUG06)	0 (17MAY06)	0 (15FEB06)	0 (04JAN06)	0
1043	4.6	1 (02AUG06)	7 (15FEB06)	0 (04JAN06)	0 (01SEP05)	2
2452	1.7	23 (03APR06)	46 (27OCT05)	85 (03MAY05)	92 (17NOV04)	62
2455	1.7	0 (03APR06)	6 (27OCT05)	8 (03MAY05)	6 (17NOV04)	5

[a] ELISA 1 is the newest sample and ELISA 4 the oldest sample of the ones shown from each animal. The values shown are the ODC% measured by the ELISA on (the sample date).

[b] The average ODC% of the last up to four samples is used to identify cattle with persistently high antibody levels in blood or milk

Table 4 Example of list of repeated antibody ELISA results from cattle categorized as high risk in *Salmonella* Dublin control herds. Risk group (R) 1 are considered most likely carrier animals whereas risk group 2 have more doubtful results and should be retested. Both groups should be managed to avoid potential spread of infection.

Animal ID	Age in years	ELISA 1 (date)[a]	ELISA 2 (date)	ELISA 3 (date)	ELISA 4 (date)	Average[b]	R
2526	2.0	98 (07NOV06)	111 (03APR06)	160 (27OCT05)	8 (03MAY05)	94	1
2545	1.9	103 (07NOV06)	91 (03APR06)	0 (27OCT05)	3 (03MAY05)	49	1
2536	2.0	122 (07NOV06)	2 (03APR06)	0 (27OCT05)	4 (03MAY05)	32	2
940	7.5	57 (02AUG06)	7 (17MAY06)	49 (15FEB06)	43 (04JAN06)	39	2
953	3.4	70 (02AUG06)	68 (17MAY06)	33 (15FEB06)	19 (04JAN06)	48	2
982	6.3	65 (17MAY06)	34 (15FEB06)	28 (04JAN06)	56 (16JUN05)	46	2
1001	5.8	95 (02AUG06)	40 (17MAY06)	46 (15FEB06)	25 (04JAN06)	52	2
1799	6.1	60 (02AUG06)	15 (17MAY06)	20 (15FEB06)	9 (04JAN06)	26	2
1858	5.4	60 (17MAY06)	13 (15FEB06)	40 (04JAN06)	58 (16JUN05)	43	2
2002	4.2	54 (02AUG06)	62 (17MAY06)	103 (15FEB06)	48 (04JAN06)	67	2
2404	2.2	55 (02AUG06)	32 (03APR06)	44 (27OCT05)	6 (03MAY05)	34	2
2458	1.7	59 (03APR06)	68 (27OCT05)	95 (03MAY05)	101 (17NOV04)	81	2
2459	1.7	50 (03APR06)	76 (27OCT05)	14 (03MAY05)	76 (17NOV04)	54	2
2484	1.6	56 (03APR06)	80 (27OCT05)	113 (03MAY05)	6 (17NOV04)	64	2
2496	1.6	57 (03APR06)	112 (27OCT05)	17 (03MAY05)	.	62	2
2508	1.5	75 (03APR06)	134 (27OCT05)	2 (03MAY05)	.	70	2
2567	1.7	55 (07NOV06)	0 (03APR06)	0 (27OCT05)	.	18	2
2658	1.3	65 (07NOV06)	96 (03APR06)	0 (27OCT05)	.	54	2

[a] ELISA 1 is the newest sample and ELISA 4 the oldest sample of the ones shown from each animal. The values shown are the ODC% measured by the ELISA on (the date).

[b] The average ODC% of the last up to four samples may aid in identifying cattle with high antibody levels in blood or milk

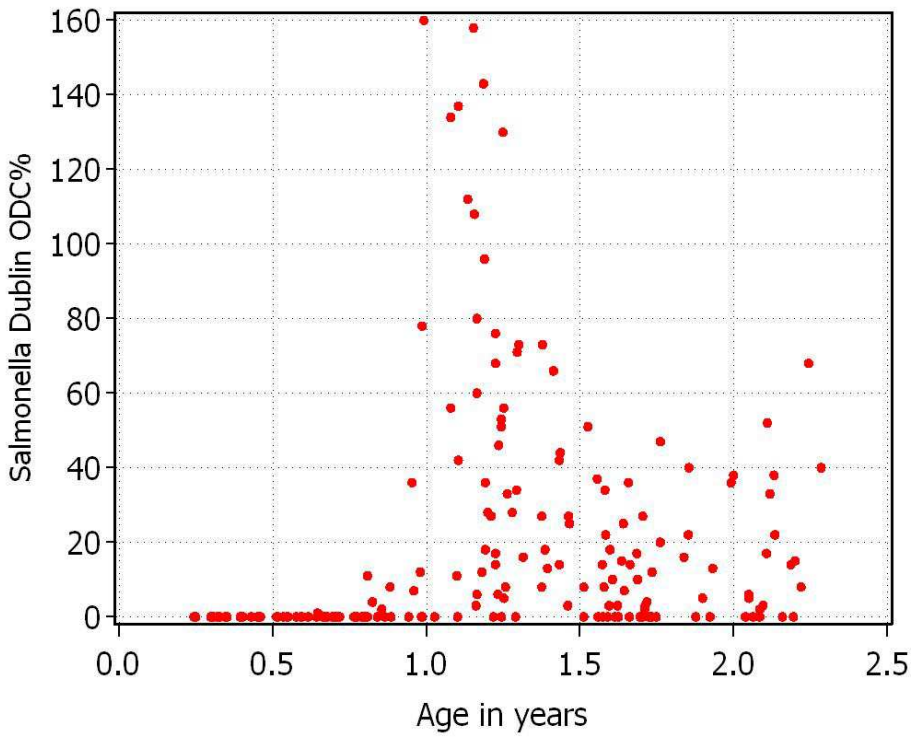

Figure 1 Example of antibody ELISA response (ODC%) vs. age in years at sampling used to evaluate progress in control of *Salmonella* Dublin in young stock and lactating cows. In this particular example there are no signs of infections having occurred in cattle below one year of age suggesting that control actions have had a good effect in this part of the herd.

Change in seroprevalence and estimated true prevalence

Overall, there was a significant reduction in prevalence from first to last sampling round in both young stock and lactating cows (Figure 2). In lactating cows, the overall estimated true within-herd prevalence across all herds was reduced from 26.9% to 2.7% (t=5.19, p<0.006) from the first to the last sampling rounds. In young stock above the age of three months, the overall within-herd true prevalence across all herds went from 15.0% to 2.6% (t=1.48, p<0.17). Only herd H did not reach 0% true prevalence in the study period.

Seroprevalence <5% was used as the criteria indicating good control of the infection. Herd H did not reach seroprevalence ≤5% in young stock in the study period. This herd reached Level 1 in the surveillance programme in February 2008. However, the young stock was not tested after December 2006, so it was not possible to say how the young stock seroprevalence developed after the project ended. Among the nine herds that did obtained seroprevalence in young stock ≤5% within the project period, the average time from initiation of control actions to when seroprevalence was ≤5% in young stock, was 13 months (std: 13), ranging from 0 to 30 months. In this project, the farmers were motivated to participate and there was frequent follow-up in the process from project leaders and local advisors. It is possible that successful intervention takes longer in herds with less motivated managers.

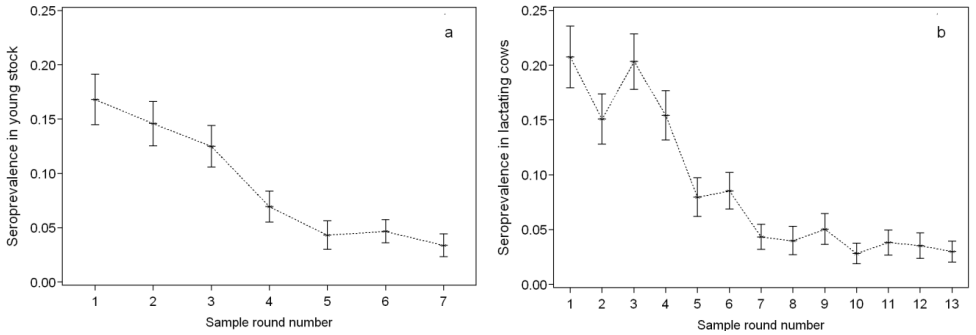

Figure 2 Development in *Salmonella* Dublin seroprevalence from first to last sampling round in a) young stock (biannual sampling) and b) lactating cows (sampling four times per year) during an intervention field study in 10 Danish dairy herds during 2003 to 2006.

Changes in surveillance programme classifications

All herds were classified as Level 2 when the study period was initiated. To reach Level 1 (most likely not infected with *Salmonella* Dublin) in the Danish surveillance classification scheme, the average of the last four bulk tank milk samples had to be below 25 ODC% in antibody ELISA, and the last sample could not have an ODC% value that was 20 ODC% above the average of the previous three samples. Furthermore, herds were not allowed to have purchased animals from herds not in Level 1. The project period officially ended in December 2006. However, because the national surveillance programme was still running, we were able to follow the herds after that. As shown in Table 1, six of the 10 participating herds changed from Level 2 to Level 1 before December 2006. Herd A reached Level 1 in April 2004, but returned to Level 2 because of too high bulk tank milk antibodies in January 2009. In the time period between these two events this farmer purchased animals from Level 2-herds on several occasions, which lead the herd to be classified as "Level 2-because of purchase/contact to Level 2". This might explain why this herd became re-infected (Vaessen et al., 1998; Nielsen et al., 2007b).

The last four herds obtained Level 1 before the end of 2008. It was an indication that infection was not stopped effectively until the end of or after the project period. The time it takes bulk tank milk antibodies to reduce to levels that will classify the herd in Level 1, after transmission of *Salmonella* Dublin among all of the young stock has ceased, can be up to around 2 years (Jordan et al., 2008).

Nine herds stayed in Level 1 at least until August 2010, which was the last sampling before submission of this manuscript. The estimated mean time herds claimed to perform control actions directed against transmission of *Salmonella* Dublin before reaching Level 1 was 24 months (std: 6). Figure 3 illustrates bulk tank milk *Salmonella* Dublin antibody levels in the participating herds from before the study period until August 2010. The individual curves show that four of the herds most likely became infected one to two years before the project started, whereas the rest of the herds had most likely been infected for at least three years before 2003.

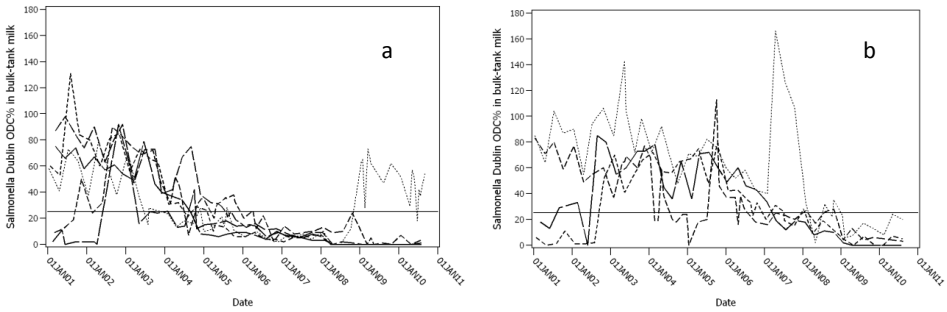

Figure 3 Bulk tank milk *Salmonella* Dublin antibody measurements in a) six herds that reached Level 1 in the surveillance programme within the study period 2003-2006. Five of these herds stayed in Level 1 at least until August 2010 and one herd had new or recurrent infection in 2009;
b) four intervention herds that reached Level 1 after the study period. The solid line indicates the cut-off value of 25 ODC% used in the surveillance programme.

Experiences from interviews

During the herd visits it became evident that it was important that herd-managers had a thorough understanding of the infection dynamics to manage infections. Control of *Salmonella* Dublin requires long-term and daily efforts, and the required actions differ from one herd to another. Consequently, communication is a major challenge in such control programmes. This includes communication between central decision makers, farmers and local advisors. Communication can be assisted by tools, which convey background information and infection status of herds and animals obtained via diagnostic test information over time such as in this study. We suggest that control strategies for *Salmonella* Dublin should contain the following five components:

a) communication;
b) reduction of transmission via changes in management and trade restrictions;
c) detection and management (or culling) of infectious animals;
d) documentation of effect of intervention;
e) continued surveillance.

Control or eradication

There was a clear reduction in the apparent prevalences and estimated true prevalences in all herds, but the apparent prevalences were not 0% in all herds at the end of the study period. The specificity of antibody ELISA is not 100%, primarily because some animals have been exposed to *Salmonella* Dublin, but have cleared the infection, or they have been infected with other *Salmonella* serovars containing cross-reacting antigens. It is therefore possible that the infection was cleared from the herds without it being reflected as seroprevalence at 0%. However, it is also possible that the used diagnostic tests have not been able to identify some infected animals, or that *Salmonella* Dublin-bacteria have remained in the environment. It is therefore not possible to deem the herds “free of *Salmonella* Dublin”, but the infection appears to currently be under control and potentially eradicated from the herd, based on bulk tank milk recordings obtained after the study period ended. Thus, the recommendation to farmers should be to continue management practises to control the infection, but testing can be reduced to a minimum after the low prevalence status has been obtained.

All cattle herds in Denmark have been included in the surveillance programme since 2002 which includes consequences upon trade with herds not in Level 1. Hence, most farmers are likely to perform some control actions when they are informed about the results and discuss these with colleagues, local advisors etc. It was therefore not possible to include infected control herds to determine, if the structured approach used in the study herds were more effective than leaving it up to self-clearance to occur. However, most of the study herds had been infected for an extended period of time before the project started. Furthermore, previous studies show that persistent infection occurs in half of the dairy herds that become infected with *Salmonella* Dublin (Veling, 2004a), and that restrictive purchase patterns are not enough in itself to stop transmission of infection within the herds (Nielsen et al., 2007b). Thus, it is not very likely that self-clearance would have occurred in the study herds without the efforts provided by the farmers in this project.

Conclusions

To our knowledge this is the first study to demonstrate that it is feasible to control *Salmonella* Dublin in endemically infected herds. Effective control of *Salmonella* Dublin in the study herds was obtained by the use of limited resources through management that focused on closing transmission routes within the herds. We found markedly reduced prevalence of antibody-positive animals in 9 out of 10 herds, and all 10 herds could be classified as most likely free of *Salmonella* Dublin infection after a follow-up period. It was, however, not possible to compare different management strategies between these herds, so the results of this study should not be interpreted as specific recommendations, but rather as an illustration of a structured approach to controlling *Salmonella* Dublin in dairy herds that appears to work. It took on average three years from initiation of control actions until monitoring suggested that *Salmonella* Dublin was no longer spreading and the seroprevalence was low in all age groups of the herd. It cannot be ruled out that more aggressive culling of high-risk animals could speed up the control. However, such a strategy might not be cost-effective. Such strategies probably have to be studied by simulation modelling, because it is difficult and resource consuming to study under field conditions.

Supplementary material related to this article can be found online at doi:10.1016/j.foodres.2011.02.027.

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PAPER XVI

Culling decisions of dairy farmers during a 3-year *Salmonella* control study

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Abstract

Salmonella enterica subsp. *enterica*-serotypes lead to periodically increased morbidity and mortality in cattle herds. The bacteria can also lead to serious infections in humans. Consequently, Denmark has started a surveillance and control programme in 2002. The programme focuses on *Salmonella* Dublin which is the most prevalent and most persistent serotype in the Danish cattle population.

A field study in ten dairy herds with persistent *Salmonella* infections was carried out over three years to gain experience with control procedures including risk assessment, targeted control actions and test-and-cull procedures. From autumn 2003 until end of 2006 quarterly milk quality control samples from all lactating cows and biannual blood samples from all young stock above the age of three months were tested using an indirect antibody ELISA. The most recent and previous test results were used to categorise all animals into risk groups. These risk groups and all individual ELISA-results were communicated to the farmers as colour-coded lists four to six times per year. Farmers were advised to manage the risk of *Salmonella* transmission from cattle with repeatedly high ELISA results (flagged as "red") or cows with at least one recent moderately high ELISA result (flagged as "yellow") on the lists. Risk management included e.g. culling or separation of the cows at calving.

We analysed culling decisions using two models. For heifers a hierarchical multivariable logistic model with herd as random effect evaluated if animals with red and yellow flags had higher probability of being slaughtered or sold before first calving than animals without any risk flags. For adult cows a semi-parametric proportional hazard survival model was used to test the effect of number of red and yellow flags on hazards of culling at different time points and interactions with prevalence in the herd while accounting for parity, stage of lactation, milk yield, somatic cell count and the hierarchical structure of the data with animals clustered at herd level.

This study illustrates how investigation of culling decisions made by herd managers when they have access to test-status of individual animals and overall apparent prevalence during control of an infection can lead to useful new knowledge. Overall herd managers were more likely to cull cattle with increasing number of yellow and red flags than animals with no flags. However, cattle were more likely to be culled with yellow and red flags during times with low or medium high within-herd seroprevalence than at times with high seroprevalence. These results are valuable knowledge for modelling and planning of control strategies and for making recommendations to farmers about control options.

Introduction

Salmonella enterica subsp. *enterica* serovar Dublin (S. Dublin) is the most commonly isolated serotype of salmonella in cattle in Denmark (Anonymous, 2010). Infected herds typically experience periodically increased morbidity and mortality among calves and abortions in adult cows (Richardson and Watson, 1971; Wray and Davies, 2000). *S*. Dublin infections in humans are rare in incidence, but invasive leading to a syndrome of sustained bacteraemia with fever, resulting in high case fatality (Helms et al., 2003). Consequently, the Danish cattle industry and the Danish Veterinary and Food

Administration started a surveillance and control campaign in cattle herds aimed at reducing *S*. Dublin prevalence to zero (or below detection limits) by end of 2014.

Control of *S.* Dublin in cattle herds is achieved through strict and persistent management procedures aimed at blocking transmission routes within the herd to stop or reduce spread of *S*. Dublin between animals in the herd, or to and from the environment (Wray et al., 1989; Jensen et al., 2004). Furthermore, purchase of replacement stock and contact to other herds need to be restrictive (Vaessen et al., 1998; van Schaik et al., 2002; Nielsen et al., 2007; Jordan et al., 2008). *S.* Dublin appears to have a tendency to produce persistently infected cattle that do not show any clinical signs and thus pose a risk of spread of infection in the herd (Richardson, 1973; Wray et al., 1989; House et al., 1993). It has been suggested that persistently infected animals have persistently high antibody responses to the infection as opposed to temporarily infected cattle, in which the level of antibodies in blood or milk will drop to low levels within two to four months after the time of infection (Spier et al., 1990; House et al., 1993). This provides an opportunity to classify individual cattle into high or low risk animals for differential management or culling decisions on the basis of repeated antibody measurements during control programmes for *S*. Dublin (Smith et al., 1992).

In intervention field studies it is often desirable to extract information about which management procedures were used by the herd managers and relate these to success rates or prevalence reductions (Jensen et al., 2004; Ellis-Iversen et al., 2008; Collins et al., 2010). In addition, drivers of decision making during control of infectious diseases are of interest (Ellis-Iversen et al., 2010). Factors affecting culling decisions can be objectively analysed when there are detailed data available about calving, movement of animals, production and health on individual animal level over an extended period of time. Survival analysis including health disorders as time-dependent variables has been suggested as most appropriate for such analyses (Beaudeau et al., 2000). To our knowledge, the effect of the salmonella status of individual animals on culling in dairy herds has never been studied before, probably because such laboratory-results are not usually available to the farmers and recorded centrally in a database. However, in the Danish *S*. Dublin control program farmers have the opportunity to request individual animal ELISA-testing through the milk recording scheme or by having blood samples collected for testing. The laboratory enters the results in the Danish Cattle Database and all tested animals are assigned a risk group at the time of sampling based on the current and previous up to four samples collected from the same individual.

This study aimed at demonstrating how culling decisions of herd managers in 10 dairy herds during a field study on *S*. Dublin control were affected by access to repeated ELISA-results and *Salmonella* risk classification from individual cattle in the herds. It was hypothesised that herd managers were more likely to cull animals that had had persistently high antibody titres in blood or milk samples than those that did not. Furthermore, investigation of whether the underlying prevalence affected the culling decisions was of interest.

Material and Methods

Selection of herds

A field study was carried out in 10 dairy herds over a period of three years to gain experience with a structured approach to control of *S*. Dublin including risk assessment followed by herd-specific targeted control actions in the herds, and test-and-cull or test-and-manage procedures. The herds were followed intensively through herd visits and frequent testing of all animals. The herds had

seroprevalences above 5% among cows at time of inclusion in the study. All 10 herds had high (>25 corrected optical density-values (ODC%)) *Salmonella*-antibody levels in bulk-tank milk measured through the Danish cattle *Salmonella* surveillance programme for one to three years prior to the onset of the study (Nielsen and Ersbøll, 2005; Nielsen and Nielsen, 2011). This strongly indicated that *Salmonella* had been present in the herds for a period and still was present in the herds at the beginning of the study period (Veling et al., 2000; Nielsen, 2003; Warnick et al., 2006). The serotype most likely to be present was *S*. Dublin even though information about relevant serotype was only available for six of the herds (five with only *S*. Dublin isolated and one with dual *S*. Dublin and *S.* Typhimurium infections). All farmers joined the study because they were motivated to actively try to eradicate the infection from their herd.

The demographics of the herds and information of management has been described in detail elsewhere (Nielsen and Nielsen, 2011). In short, herd size went from an average of 97 cows (95%CI: 75-119) at the beginning of the study period to an average of 123 cows (95%CI: 97-150) at the end of the study period. One was a Jersey herd and nine were Danish Holstein breeds. Eight of the herds were conventional, one was organic during the first 1½ year of the study period, and one herd was organic throughout the study period from mid 2003 to end of 2006.

Sampling of individual cattle

From autumn 2003 until end of 2006 milk recording samples from all lactating cows were collected every three months and blood samples from all young stock above the age of three months and until first calving were collected twice per year. The samples were tested using an indirect ELISA that measured antibodies directed against O-antigens of *Salmonella* serogroup-D. S. Dublin is with very few exceptions the only serogroup-D *Salmonella* type isolated in cattle. The test results were used to categorise all animals into risk groups based on current and previous test results, and the risk groups and ELISA-results were communicated to the farmers four to six times per year, usually one month after each new testing round. The test procedures and validity estimates are described in the serological methods section, and the criteria for the risk groups are described in the section about risk groups and seroprevalence below.

Farmers were advised to consider culling cows with repeatedly high ELISA results, in particular if they were not able to manage the risk of transmission of bacteria by isolating the high risk cows from young calves during and after calving and from other cows in the calving area. However, farmers were advised to make their choice of control procedures specific to their own herd instead of following general advice, and they were asked to regularly evaluate the progress and adjust their decision-making if necessary. Thus, it was not possible to classify the herds according to a certain set of management procedures.

Serological method

The in-house ELISA used for the blood and milk samples at Eurofins Laboratory (Holstebro, Denmark) has been described in detail elsewhere (Nielsen and Ersbøll, 2004; Nielsen et al., 2004). The ODC% was calculated for each sample as follows:

$$ODC\% = \frac{\left(\overline{OD}_{sample} - \overline{OD}_{neg\ ref}\right)}{\left(\overline{OD}_{pos\ ref} - \overline{OD}_{neg\ ref}\right)} * 100\%$$

where $\overline{OD}_{sample}$ is the mean value of two test wells, and $\overline{OD}_{negref}$ and $\overline{OD}_{pos\ ref}$ are the mean values of four negative and four positive reference wells in the ELISA plates. The scale of ELISA values goes from 0 to approximately 200 ODC% and can be interpreted as a semi-quantitative scale of the concentration of antibodies in the sample. Although the antigen used in the assay was developed to detect antibodies directed against *S*. Dublin, cross-reactions with other serotypes of *Salmonella* are known to occur (Konrad et al., 1994). Under Danish conditions it would mainly be *S*. Typhimurium-serotypes that might cause cross-reactions.

The sensitivity (Se) of single measurements at animal level has been estimated to be approximately 50% and the specificity (Sp) approximately 98% at cut-off 50 ODC% in cattle above 300 days old for the serum test (Nielsen and Ersbøll, 2004). For the milk ELISA, Se was estimated to be approximately 43% and Sp approximately 90% (Nielsen, 2003). The Se is much higher (94%) for actively shedding carriers (Veling et al., 2000). However, the test sensitivity and specificity estimates and the predictive values for these tests are not essential for this study, because conclusions were not drawn about true infection status of the tested animals nor the effect of culling animals classified as high-risk on success or failure of control.

Risk groups and seroprevalence

The criteria of the serologically determined risk groups were modified from recommendations in previous experimental and field studies (Smith et al., 1989; Spier et al., 1990; House et al., 1993). Heifers and cows were categorised as high risk indicated by a "red flag" on the result lists provided to the farmers, if they had at least two samples above 80 ODC% with a minimum of 120 days in between, the most recent sample was above 80 ODC% and the average of the last up to four samples was above 80 ODC%. The animals were categorised medium risk indicated by a "yellow flag" if the most recent ELISA and the average of the last up to four samples were above 50 ODC%, but not high enough to be categorised as high risk. Animals with ELISA values below 50 ODC% in the most recent sample did not have any colour indicators on the decision support lists.

Two datasets were created for further analysis, one for heifers (female young stock) and one for adult cows. This split of data was used because milk production data could only be included for lactating cows. In the heifer dataset, the within-herd prevalence of *Salmonella* was calculated as the number of animals with yellow or red flags out of all tested animals in the herd in the relevant sampling round (twice per year). The within-herd prevalence was considered low if <5% (the mean within-herd prevalence) and high if ≥5%. In the cow-dataset, the prevalence was calculated as the number of cows with yellow or red flags out of all tested cows in the herd in the relevant sampling round (four sample rounds per year). Prevalence was categorised as low if <5%, medium if between 5 and 15% and high if >15%.

Data management

Heifer dataset

The dataset of heifers included animals that had been sampled at least three times and was constructed with one observation per animal indicating herd-id, animal-id, number of red and yellow flags, and within-herd seroprevalence at the last sampling date before culling or first calving, and whether or not the heifer was sold or slaughtered before the first calving.

Cow dataset

The adult cow dataset was constructed with one observation per sampling interval. The first interval went from the first ELISA test date to next ELISA test date (or in case the cow was culled before the next sampling round, the last date of the interval was set to be the culling date). The next interval went from the second ELISA test date to the next ELISA date and so forth. Thus, the cows entered the study on the first date they were ELISA tested. Cows were either censored on the last ELISA test date plus 92 days, if they were not culled within this period, or were set to have a failure ("culled" implying sold or sent to slaughter) and left the study on the date of culling. For each interval the relevant *Salmonella* risk group was given. Cumulative numbers of red and yellow flags up to and including the most recent ELISA date was counted for each cow-interval.

Confounding variables in cow dataset

Milk yield was recorded 11 times per year through a milk recording scheme at which kilograms of milk, percentage of fat and percentage of protein were determined. Energy corrected milk yield (ECM) was calculated on each milk quality control test date as (kg of milk × (383 × fat% + 242 × protein% + 780.8))/3140 (Nielsen et al., 2009). The following expected confounding variables were constructed for each of these intervals: The mean energy corrected milk yield (mean-ECM) and mean of the natural logarithm to the somatic cell counts (mean-lnSCC) measured in each interval based on all milk recordings performed in that interval; days in milk (DIM) and parity on the first day of the interval.

Six two-level predictive models for ECM were constructed for first, second and third and higher parities and for each of the two types of breed groupings in the study herds (large breeds (9 herds) and Jersey (1 herd), respectively. The models predicted the test day ECM including Wilminks correction as DIM * exp (-0.065*DIM) (Silvestre et al., 2006). The mean deviation from the predicted milk yield (in %) according to the models were included in the dataset as a potentially confounding variable (mean-pctECM).

Statistical analysis of heifer data

A two-level hierarchical logistic regression model was used to analyse the data on heifers to account for the clustering of animals in herds. The analysis was performed in STATA® IC/11 (StataCorpLP, College Station, Texas, USA) using a subject specific model (xtmelogit). Outcome in the model was a binary variable indicating whether the heifer was culled before first calving or not. Herd was included in the model as a random effect to account for clustering of animals at herd level. Forward stepwise inclusion of variables was used to assess significance of the main effects and interactions of all explanatory variables. The model was fit using maximum likelihood estimation. The model fit when allowing for random slopes of the herd effect was assessed by comparing log-likelihood to the final model without random slopes.

Statistical analysis of cow data

All the statistical analyses of cows were performed in STATA® IC/11. The time to culling in adult cows was analysed using a semi-parametric survival model (Cox proportional hazards model). Efron's method was used to handle ties in the data (multiple culling events on the same end of study days for cows). The hierarchical structure of the data with animals clustered at herd level was accounted for by including herd as a gamma distributed shared frailty in the proportional hazards model. The estimation of the shared frailty was done using a penalised likelihood function (Dohoo et al., 2009).

Initially mean-ECM, mean-lnSCC, DIM and parity were forced into the model due to expected strong confounding effects. The optimal functional form of continuous and discrete predictors with more than 10 levels was determined by the use of fractional polynomials and evaluation of lowess smoothed graphs of Martingale residuals (Royston and Sauerbrei, 2008). The fractional polynomial form (up to 4 terms) which best fit the data was forced into all consecutive models to control for confounding.

Then a stepwise forward selection procedure was used to test the rest of the explanatory variables including possible two-way interactions between the explanatory variables of interest in the model. All effects were evaluated at a 5% significance level. Inclusion of time-varying variables was used at the end of the modelling procedure where it was evaluated as necessary by assessment of significance levels and differences in log-likelihood between subsets of models.

The assumption of proportional hazards was evaluated graphically for the categorical variable year and by graphical and statistical test evaluation of Schoenfeld residuals for continuous variables included in the final model. These procedures evaluated whether of not there was evidence that some hazard ratios, conditional on the frailty effect (i.e. the effect of a change in the number of flags within a herd), were non-proportional (i.e. changed over time). The assumption of independent censoring was evaluated by sensitivity analysis comparing scenarios with complete positive and negative correlations between censoring and culling. The overall fit of the model was assessed by graphical evaluation of the Cox-Snell residuals (Dohoo et al., 2009). Finally, we checked for outliers by plots of deviance residuals vs. time and influential points by plots of score residuals vs. time.

Results

Results of logistic analysis of culling of heifers

The risk group variable was categorised into a three-level flag variable counting the number of yellow and red flags. Only 76 out of the 1,491 heifers included in the study had yellow or red flags. Risk flag=0 indicated no yellow or red flags, risk flag=1 indicated one or more yellow flags and risk flag=2 indicated one or more red flags. Within-heifer prevalence was categorised as low if below, and high if above or equal to 5% (the mean heifer prevalence). There were only two heifers with red risk flags when the within-herd prevalence was low. In general there were more animals included in the dataset in 2005 and 2006 due to the criteria that the animal had to have been tested at least three times to be included. Table 1 shows the distribution of the categorised prevalence and risk flag variables in culled and non-culled heifers. In the initial univariable cross-tabulations the risk of culling appeared to be significantly higher with increasing risk flag number (χ=33.8, p<0.0001).The results of the final multivariable model are shown in Table 2. Heifers with one or more yellow flags had 2.7 (95%CI: 1.3-5.8) times higher odds of being culled, and heifers with one or more red flags had 11.5 (95%CI: 4.7-28.3) times higher odds of being culled than heifers with no flags. Furthermore, heifers

had twice the odds of being culled when prevalence was low as opposed to when prevalence was high (in the table OR for high prevalence=0.5, p=0.009). However, the risk of culling did not change between years. Fig. 1 illustrates the associations between having yellow or red risk flags and the probabilities (shown both as raw proportions in the dataset and model predicted probabilities) that a heifer was culled before the first calving during low and high within-herd prevalence.

Table 1 Distribution of culled and non-culled heifers in different years, within-herd prevalence groups and *Salmonella* risk groups in 10 dairy herds during a three year *Salmonella* control study

Explanatory variables	n	Culled before first calving (%)	Not culled before first calving (%)
Number of risk flags			
Zero flags	1415	145 (10.2%)	1270 (89.8%)
One or more yellow flags	52	10 (19.2%)	42 (80.8%)
One or more red flags	24	11 (45.8%)	13 (54.2%)
Within-herd prevalence groups			
Low prevalence (<5%)	909	119 (13.1%)	790 (86.9%)
High prevalence (≥5%)	582	47 (8.1%)	535 (91.9%)
Year			
2004	141	13 (9.2%)	128 (90.8%)
2005	500	57 (11.4%)	443 (88.6%)
2006	850	96 (11.3%)	754 (88.7%)

Results of survival analysis of culling of adult cows

The distribution of observations in each of the prevalence-flag groups are shown in Table 3. In Fig. 2 the functional form of the continuous confounding variables and log hazards of culling in the cows are illustrated. A total of 4400 cows were included in the dataset. Some cows were represented in several prevalence-flag groups, because they changed test status or the herd changed seroprevalence as time went by in the study period. The variables included in the final survival model are presented together with parameter estimates, standard errors, hazard ratios and p-values in Table 4. The effects of three parameters varied with time: 0 flags and >5 flags in medium prevalence and 0 flags in high prevalence. The time effects gave similar results when modelling the variation over time as linear and log-linear, so for simplicity it was decided to base the results on the linear form. Fig. 3 illustrates the hazard ratios for each flag group relative to the reference group with 0 flags within each prevalence group at the median number of study days for the time-varying prevalence-flag groups. For instance, cows with >5 flags had 2.6 times higher hazard of being culled than cows with no flags during low prevalence periods and this remained constant over the study period.

Table 2 Parameter estimates *(β),* standard error (S.E.), odds ratios (OR), 95% confidence interval of OR and significance level (*P*) in the final logistic regression model for probability of culling in heifers in 10 dairy herds during a three year *S*. Dublin intervention study. Risk flags indicate if heifers have been assigned medium (yellow flags) or high (red flags) risk for spreading *Salmonella*.

Explanatory variables	Estimate *(β)*	S.E.	OR	95% CI of OR	*P*
Intercept	-2.10	0.24			-
Risk flags					<0.0001
Zero flags	0		1		
One or more yellow flags	1.00	0.39	2.7	1.3-5.8	
One or more red flags	2.44	0.46	11.5	4.7-28.3	
Prevalence groups					0.009
Low prevalence (<5%)	0		1		
High prevalence (≥5%)	-0.79	0.30	0.5	0.3-0.8	
Random effect of herd					
Variance component estimate	0.38	0.22			

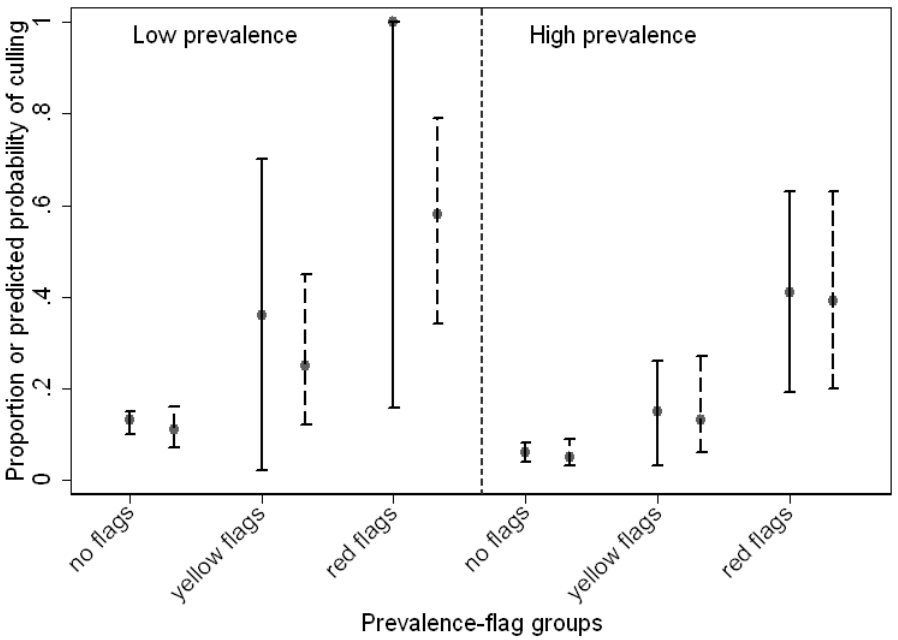

Figure 1 Proportions in the raw data (solid lines) and predicted probabilities (dashed lines) with 95% confidence intervals from a logistic analysis of heifers being culled before the first calving in different *Salmonella* risk flag groups under low (<5%) and high (≥5%) within-herd seroprevalences. There were only two heifers with red flags in the low prevalence group and both were culled, thus the exact one-sided 97.5% confidence interval was calculated for this proportion.

The functional forms of the confounders illustrated in Fig. 2 were evaluated to be reasonable. For instance they showed that the risk of culling increased during the lactation (DIM) and with increasing somatic cell count (lnsccc), and risk of culling decreased with increasing milk yield (ECM) and the more the milk yield exceeded the expected milk yield for each cow (pct-ECM).

The difference in risk of having >5 flags vs. no flags during medium high prevalence times changed over the study period from no difference (HR=0.1, Table 4) at the beginning of the study period to more than three times the hazard (HR=3.3, Fig. 3) at the medium number of study days for that group. In contrast, cows with >5 flags were not more likely to be culled than cows with no flags during periods with high prevalence in the herd (HR=0.4, Table 4) and this difference in risk did not change significantly over time.

The model fit as assessed by plots of Shoenfeld residuals for continuous variables did not raise concerns (data not shown). Neither did plots of the Cox-Snell residuals for the overall fit of the model (data not shown). We did not find influential outliers in the data. The assumption of independent censoring was evaluated to be reasonable by sensitivity analyses of correlations between censoring and culling.

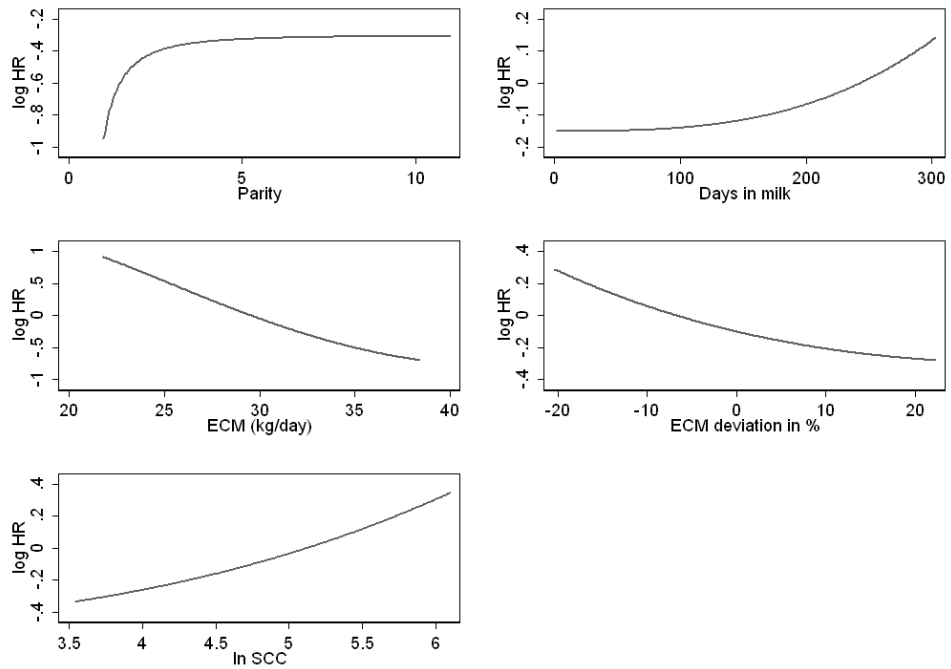

Figure 2 Functional forms of the relationships between continuous confounders and the log hazard ratio (log HR) of culling in adult cows. The confounders were: Parity (1 to 11), number of days from calving (Days in milk), energy corrected milk yield (ECM), deviation in % from the expected energy corrected milk yield adjusted for breed, parity and days in milk (ECM deviation in %) and the logarithm of the somatic cell count in milk (ln SCC).

Table 3 Distribution of cows in twelve *Salmonella* prevalence-risk flag groups in the dataset used for survival analysis of culling of cows during a three year intervention study in 10 dairy herds. Flags are the cumulative number of yellow (medium risk) or red (high risk) flags for each animal in the given time-interval.

Prevalence-flag group	n*	culled	Mean number of days spent in that prevalence-flag group
Low prev, 0 flags	2172	540	309
Low prev, 1 flag	24	3	87
Low prev, 2-5 flags	66	11	116
Low prev, >5 flags	25	7	87
Medium prev, 0 flags	1603	277	241
Medium prev, 1 flag	75	4	100
Medium prev, 2-5 flags	145	27	127
Medium prev, >5 flags	41	19	171
High prev, 0 flags	1090	195	284
High prev, 1 flag	411	34	121
High prev, 2-5 flags	273	56	200
High prev, >5 flags	30	8	206

*n= number of cows represented in each group. Cows can be represented in several different groups over time.

Discussion

To our knowledge this is the first study to evaluate the effect of individual animal level *Salmonella*-test status on culling probabilities of heifers and cows in dairy herds that are attempting to control *Salmonella*-infection. The cut-off values used for the classification of the animals were not decided by the authors aiming to be used in the study. They were used by the classification system set up in the Danish Cattle Database. In this study the classifications (yellow and red flags) that were communicated to the farmers during the study period were simply used to analyse how the farmers made decisions based on these results. To our knowledge it is not known how large a proportion of cattle in the red or yellow flag groups are truly infected or infectious. However, one study found that three out of nine animals with repeated antibody measurements that would lead to a red flag in this study carried the infection in internal organs, but none of them shed bacteria in faeces or milk (Lomborg et al., 2007).

There were high hazard ratios for >5 flags in the low prevalence group and 2-5 flags in the medium prevalence group, but not in the high prevalence group. One flag appeared to be protective against culling in the high prevalence group. Overall, there appeared to be decreased hazard ratios for culling in the high prevalence groups. Exceptions to this were medium and high prevalence groups with no flags. Due to the time-varying effect in these groups the hazard ratios went from low to high over the course of the study.

Table 4 Parameter estimates (β), standard error (S.E.), hazard ratios (HR), 95% confidence intervals for HRs and significance level (*P*) in the final proportional hazards survival model for probability of culling in adult cows in 10 dairy herds during a three year *S*. Dublin intervention study. Risk flags indicate the number of times heifers have been assigned medium or high risk of spreading *Salmonella*.

Predictors	Estimate *(β)*	S.E.	HR	95%CI of HR	*P*
Year					< 0.0001
2004	0	-	1		
2005	-0.74	0.13	0.5	0.4-0.6	
2006	-0.17	0.12	0.8	0.7-1.1	
Prevalence-flag groups					< 0.0001
Low prev, 0 flags	0	-	1		
Low prev, 1 flags	0.28	0.58	1.3	0.4-4.2	
Low prev, 2-5 flags	0.02	0.38	1.0	0.5-2.2	
Low prev, >5 flags	0.94	0.51	2.6	0.9-7.0	
Medium prev, 0 flags	-0.89	0.17	0.4	0.3-0.6	
Medium prev, 1 flags	-1.28	0.59	0.3	0.1-0.9	
Medium prev, 2-5 flags	0.55	0.23	1.7	1.1-2.7	
Medium prev, >5 flags	-2.14	1.11	0.1	0.0-1.0	
High prev, 0 flags	-1.17	0.21	0.3	0.2-0.5	
High prev, 1 flags	-1.63	0.29	0.2	0.1-0.3	
High prev, 2-5 flags	-0.61	0.21	0.5	0.4-0.8	
High prev, >5 flags	-0.91	0.42	0.4	0.2-0.9	
Time effect per 100 days "Medium prev, 0 flags"[a]	0.15	0.03	1.2	1.1-1.2	<0.001
Time effect per 100 days "Medium prev, >5 flags"	0.40	0.13	1.4	1.2-1.6	0.002
Time effect per 100 days "High prev, 0 flags"	0.12	0.04	1.1	1.0-1.2	0.005
Effect of continuous confounding variables[b]					
$LnSCC^3$	0.004	0.0003			0.000
PctECM	9.08	5.78			0.116
$PctECM^{0.5}$	-19.51	6.97			0.005
$PctECM^2$	-0.15	1.15			0.898
$(Days\ in\ milk/100)^3$ (dimcub)	0.01	0.003			0.001
$1/(Parity^2)$ (parity_invsq)	-0.65	0.14			0.000
LnECM	194.76	24.85			0.000
$LnECM^2$	58.22	7.91			0.000
$ECM^{0.5}$	-576.55	76.18			0.000
$LnECM^{0.5}$	66.59	8.82			0.000
Frailty effect of herd	0.14	0.07			

[a] the time effect per 100 days is the estimate adjusting the main effect of the relevant prevalence-flag group by study days

[b] HR and 95%CIs for HRs not shown for confounding variables

The fact that increasing number of risk flags was associated with increased risk of culling was expected, because in the study farmers were advised to consider culling these animals as part of the control strategy, in particular if they were not able to otherwise manage the risk of *Salmonella*-transmission from the high risk animals by isolation or separation. However, the analyses of the data provided a more nuanced culling pattern, in that farmers were more hesitant to cull animals with risk flags during periods with high within-herd prevalence than during periods with low within-herd prevalence. One explanation for this could be that when the prevalence is high the number of animals with risk flags is higher than when prevalence is low, and it is not feasible to cull too many heifers and cows at the same time in a herd without losing too much of the production capacity and having to purchase replacement heifers. This is important to take into account when evaluating potential control strategies for instance in simulation models. The herds were followed using four annual bulk-tank milk measurements from 2007 to 2010 after the control period ended (data not shown), and in all herds repeated individual ELISA results indicated that the herds were able to stop transmission of *Salmonella* despite the fact that culling was not used consistently in the control period (Nielsen and Nielsen, 2011).

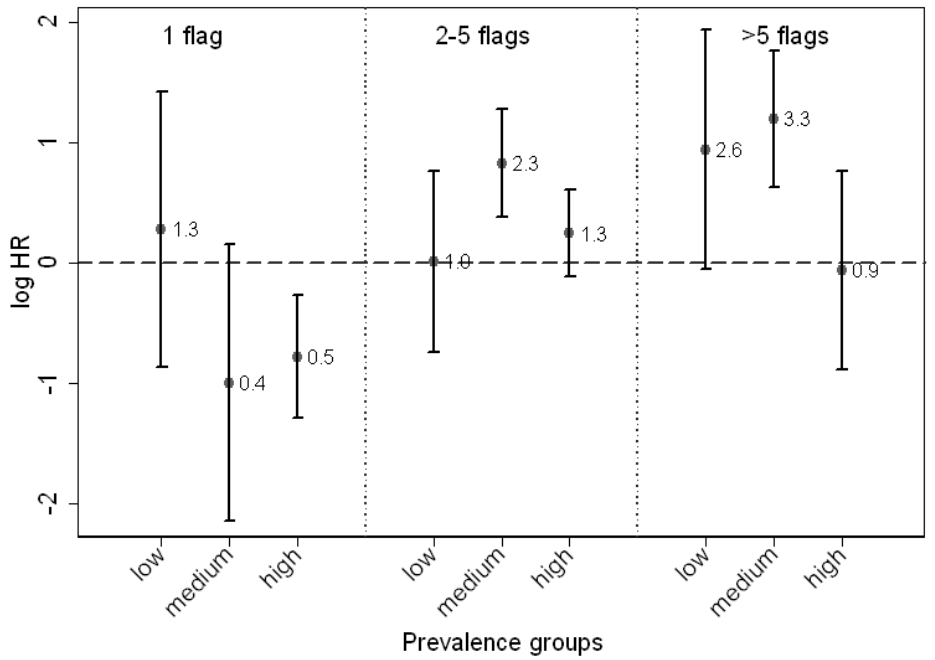

Figure 3 Log hazard (log HR) of culling in all *Salmonella* prevalence-flag groups with 95% confidence intervals at the median number of study days for the time-varying prevalence-flag groups. The numbers next to the dots on each line show the corresponding hazard ratio of the prevalence-flag combination compared to the reference group "0 flags" for each prevalence level.

In our survival model, herd was included as a frailty (random effect) and the model fit improved by keeping it in the model. This can be interpreted as overall differences between herds in general culling strategies. Investigating differences among herds in the effects of prevalence-flag groups

would have required fitting a model with up to 11 additional variance components (random slopes). The data would not support this expansion of the model.

Survival analysis with implementation of time-varying effects of health conditions has been suggested as the most appropriate method for analysis of farmers' culling decisions (Beaudeau et al., 2000). Parity, mastitis, teat injuries, poor milk yield and to some extend metabolic, reproductive and foot disorders have been shown to be drivers of culling (Beaudeau et al., 2000; Cramer et al., 2009). In this study we took into account parity, lactation stage, somatic cell counts and milk yield, both as absolute yield and as the deviation from the average of the herd mates at the same parity and lactation stage. We were not able to include other disorders due to lack of reliable data for those.

Care has to be taken in the interpretation of the results, because as shown in Table 1 and Table 3 some flag or prevalence-flag groups had few observations. We have included 95% confidence intervals in Figs. 1 and Fig. 3 to illustrate the uncertainties of the estimates. Some of the prevalence-flag groups in Fig. 3, which show culling hazard estimates at medium number of study days for each prevalence-flag group, have reasonable narrow confidence interval and conclusive estimates. For cows there was a protective effect of having one flag in the medium and high prevalence groups. This effect became even more pronounced as number of study days increased (results not shown). The explanation for this could be that during the study farmers became aware that it might be a good idea to wait and see if the next ELISA-measurement would confirm the status of the cow as being a high risk animal, or if it was just a temporary increase in antibodies that caused the first flag. Having 2-5 risk flags was associated with increased risk of culling in the medium and high prevalence groups, but not in the low prevalence group. This group only had 11 culled cows and 66 cows in total across all herds, so it is difficult to say if it is due to poor sample size that we were not able to show an effect. Cows having >5 risk flags had higher risk of culling compared to cows with no flags in the low and medium prevalence groups, but not in the high prevalence group. The high prevalence group only included 30 cows out of which 8 were culled across all 10 herds. Culling of high risk cows has been recommended during the control period to avoid re-infection of the increasingly susceptible herd (Spier et al., 1990; House et al., 1993; Jensen et al., 2004), but if there are too many of them on the list it might not be financially wise to cull them all at the same time.

In Denmark, all farmers can order single or repeated ELISA measurements for *Salmonella* antibodies on all or selected animals and have easy access to the results either electronically or by letter. This study illustrates behavioural patterns of farmers provided with such decision tools during a control programme. The herds were selected to participate in the study because they had expressed interest in participating either directly or through their local veterinary advisors. Thus, these herds are representative of herds with motivated farmers or herd managers that choose to actively intervene against *Salmonella* through management and testing strategies. Hence, they might not be representative of farmers that are less encouraged to control the infection, but might be more or less forced to for instance through national legislation.

According to a simulation study about optimal control strategies for *Salmonella* in cattle one of the most effective ways to achieve national prevalence reduction is to reduce the time period a herd is infected (Jordan et al., 2008). It is supported by literature to be a rational approach to *Salmonella* control in cattle herds to try to reduce the spread of the infection through separation and hygienic routines instead of initiating a test-and-cull strategy when there is still widespread infection among

the animals and environment in the herd (Wray et al., 1989; Wray and Davies, 2000). After this control study ended, the recommendation to only use culling according to repeated ELISA-measurements in the face of low prevalence among young stock became incorporated in the Danish *Salmonella* Dublin control campaign.

Conclusion

Using a two-level multivariable logistic analysis model for culling of heifers and a Cox proportional hazards survival model for culling of cows we were able to demonstrate that farmers were more likely to cull animals detected as high risk for *Salmonella* in 10 dairy herds during a 3-year control period. However, the culling risk of cows was strongly influenced by the within-herd seroprevalence in the herd probably due to the fact that too many animals would have to be culled during high-prevalence times if this was not taken into account when making culling decisions. These results are valuable knowledge for modelling of control strategies and for making recommendations to farmers about control options. Furthermore, this study illustrates a statistical method applied to data from a field study to explore how culling decisions of farmers are affected by access to knowledge about the test-status of individual animals during control.

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